REPRODUCTIVE INTERFERENCE AMONG COCCINELLIDS IN THE GENUS *COCCINELLA*: IMPLICATIONS FOR THE IMPACT OF NON-INDIGENOUS SPECIES

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Leonard Michael Stellwag

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Leonard Michael Stellwag, Ph. D.

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Reproductive interference is a powerful force that shapes ecosystems, influences species' distributions, and can contribute to the success of invasive species. I examined the role of hybridization and satyrization, two forms of reproductive interference, in the interactions between two North American native and one introduced species of lady beetle (Coleoptera: Coccinellidae). Through controlled pairings, I determined that hybridization between the introduced species, *Coccinella septempunctata* (C7), and two congeneric native species, *C. novemnotata* (C9) and *C. transversoguttata* (CT), is not possible and cannot be contributing to the decline of the native species. However, I quantified a significant fitness cost to C9 females for mating with a C7 male and a significant reduction in the propensity of both male and female C9 to mate with a conspecific after non-sexual encounters with C7. Additionally, I used the evolutionary relationships of each species pair, allopatric (C7/C9) or sympatric (C7/CT and C9/CT) to test the hypothesis that closely related allopatric species are more likely to intermate than sympatric congeners due to lack of recent interactions to reinforce isolating barriers. Not only were C7/C9 pairs more likely to mate than the sympatric pairs, but C7 was involved in a significant proportion of all heterospecific copulations. C7 may, therefore, impose a greater cost to C9, its allopatric congener than CT through reproductive interference. I conclude that hybridization can not be influencing the decline of these two native species, but satyrization of C9 by C7 does impose a cost to the native, but the extent of which it occurs in nature remains

unknown. That C9, but not C7, was less likely to mate with a conspecific after repeated non-sexual encounters with a heterospecific raises interesting questions about the role of this behavior in the decline of C9 and may represent a cost to the evolution of satyrization-resistance. This has important implications for other declining native species. As the native becomes rarer and the invasive more common, the native will encounter heterospecifics more often than conspecifics. If this results in a decreased propensity to mate with a conspecific, already diminished populations may have little chance of recovery.

BIOGRAPHICAL SKETCH

Leonard Michael Stellwag was born in Muncie, IN and raised by his mother, Shirley, along with his two brothers, Brian and Dwon, and his cat Kitty Tom, who was born in the same year as he. His love of animals and nature was apparent from a very young age and for many years he aspired to become a veterinarian. He attended Ball State University in Muncie, IN and was taking courses to prepare for veterinary school when he discovered psychology and became captivated with gendered human behaviors related to sex, gender, and sexuality. He completed a Bachelor's of Science degree in Psychological Science with minors in Women's Studies and Counseling Psychology and honors from the Ball State Honors College and Psychology Department in 1998, becoming the first person in his family to receive a college degree. Upon graduation Leo decided to take a few years off before going to graduate school and accepted a promotion with Franklin Covey, relocating to Boston, MA. He had the good fortune to live near the iconic Walden Pond in Concord, MA and spent a lot of time there swimming, fishing, reading, and reflecting away from the hectic Boston scene. It was the time spent at Walden Pond that inspired Leo to pursue a Ph.D. in biology, a he established in 2001. Between 2001 and 2003 he took introductory courses at the University of Massachusetts, Boston and Quincy College to fill in gaps from his education in the social sciences. During 2004-2007 he completed a Master of Science in Biology at Ball State University under the guidance of entomologist, Dr. Gary Dodson, conducting research on the mating system of a native crab spider. Inspired by his advisor's love of insect behavior, he decided to pursue a degree in Entomology at Cornell University and in the Fall of 2007, 6 years after setting the goal, Leo was finally able to begin a PhD program in the biological sciences. Throughout his tenure at Cornell, Leo has pursued his love of teaching through teaching assistantships for a variety of courses; frequent guest lectures on biology, entomology, and gender and sexuality; completion of the Cornell Graduate Teaching Assistant Fellowship program through the Center for Teaching Excellence; and designing and teaching his own course on the Biology of Sex at Ithaca College for several semesters. During the past 8 years Leo has also faced many significant challenges and a few seemingly

insurmountable obstacles, but with the encouragement of friends, family, and the unwavering support of his advisor, Dr. John Losey, was able to finally achieve the goal he established nearly 15 years ago and became the first person in his extended family to complete a terminal degree. Leo plans to work in academia, continuing to teach undergraduate students about the fascinating world of biology and animal behavior. He hopes to get a job somewhere warm and sunny.

Dedicated to my mother, my chosen family, and all the teachers who never once doubted my potential, offering encouragement as I fought my way to and through higher education.

And to Roxy, the sweet and silly bullboxer who rescued me in September 2014.

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I am forever indebted to my committee chair, Dr. John Losey, whose kindness, generosity, guidance, and humor made the completion of this dissertation possible. I am certain there were times when he believed in me more than I did in myself and he always had the right and witty words (usually involving puns) to get me back on track. He is a true advocate and deeply cares for his students and I am deeply grateful for the opportunity he gave me.

My committee members have also been very supportive. Dr. Jennifer Thaler, ecologist extraordinaire, asks tough questions, gives great advice and has been a steady supporter from the beginning. Her comments are challenging and on point, something I very much appreciate in the feedback I receive about writing, it's the only way I improve. Dr. Toni DiTommaso joined my committee relatively recently and provided thoughtful feedback on the third and fourth chapters from the perspective of a weed biologist. I think its easy for some people to forget that there are some significant similarities between the ecologies of plants and animals. For example, I found more papers on plants than animals when I was researching reproductive interference by invasive species!

There are a few other key people in the Entomology Department who have played a significant role in this dissertation and my time as a graduate student at Cornell. Dr. Cole Gilbert was very helpful early in my tenure and provided valuable feedback, guidance, and resources. Cole has a way of making everything sound really fascinating so I always enjoyed our interactions and walked away feeling even more interested. The staff associated with The Lost Ladybug Project, Dr.'s Leslie Allee and Rebecca Rice Smyth have provided numerous types of valuable support and plenty of encouragement. I have enjoyed working with them on projects associated with the LLP and promise to submit photos of any ladybugs I see in my journeys. Dr. Todd Ugine, who joined the Losey lab a couple years ago, has been tremendously helpful in many ways, offering

statistical advice, providing specimens or other supplies, and just being a nice and decent person to be around. His enthusiasm is contagious and I enjoyed watching him do research, he's a force to be reckoned with! It always seemed like he had a half dozen experiments going on at any given time. The Losey lab is very fortunate to have him.

I would also like the thank the various student lab assistants who worked in the Losey lab during my tenure and in particular, Ting Yuet. Ting has since graduated from Cornell, but she was one of those undergraduates that you selfishly don't want to graduate because that means they will leave you. I mourned the loss of Ting as a lab assistant for a good year after she left. She's the kind of undergraduate who gets mistaken for a grad student because of their dedication to whatever task they are assigned. Ting was not only a good worker, but a fun person to have in the lab. Every lab needs a Ting, she made work feel less like "work".

Finally, there are a few people who played a very significant role in the achievement of this goal, something I set out to accomplish nearly 15 years ago. My ex-wife, Carolyn Coogan, supported me in several ways while I completed a master's degree at Ball State just so I could get into a PhD program. I would not be writing this now if she hadn't made certain sacrifices. My first academic father, Dr. Gary Dodson at Ball State continues to be an influence on me. On more than one occasion, when confronted with a challenging research or teaching-related question, I have asked myself "what would Gary do?". Gary inspired my initial interest in the behavioral ecology of insects and continues to inspire my teaching pedagogy. Jan Allen, Associate Dean for Academic and Student Affairs at the Cornell Graduate School, who started Dissertation and Thesis Writing Boot Camps several years ago, has been an amazing resource for me since I enrolled in the summer 2013 boot camp. Jan is incredibly knowledgeable and genuinely cares about students. I believe Jan, through boot camp and her optimistic encouragement, may have saved my dissertation. Last, but by no means least, I would like to acknowledge my mother, Shirley Markley, who saved my educational career from disaster during my war with long

division in fourth grade. I've been told that I cried many a tear over long division and insisted that it was impossible for me to learn, that I wanted to give up. I'm glad she didn't let me. My mother made many sacrifices so that I could have a chance at a decent education, an opportunity most people in my family never get. Most importantly, she taught me that if I worked hard and believed in myself, I could accomplish amazing things. Thanks to her, after I mastered long division I never doubted myself again. What an amazing gift to give to a child.

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CHAPTER 1

INTRODUCTION: NATIVE LADY BEETLE DECLINE IN NORTH AMERICA

Coccinellids, commonly called lady beetles, ladybugs or ladybirds, have been the subject of much discourse and research in the past 20 years since the discovery that several native species have experienced precipitous declines in North America (Wheeler and Hoebeke 1995; Harmon et al. 2007) and elsewhere (Adriaens et al. 2008; Brown et al. 2011; Roy et al. 2012) leading some to fear local extirpations in areas where they were once very abundant (Stephens 2002; Majerus et al. 2006). Out of 4200 coccinellid species, approximately 500 of which live in North America north of Mexico (Gordon 1985; Hodek et al. 2012), many of which are considered beneficial because of their effectiveness as predators of economically important agricultural pests, mainly Homopterous insects and mites (Iperti 1999). In addition to their role in biological control, coccinellids may be useful bioindicators due to their ubiquity in all ecosystems of the world (Hodek et al. 2012), vulnerability to environmental changes, and sensitivity to anthropogenic influences such as changes in land use and pollution (Iperti 1999).

Rapid declines of some populations of indigenous coccinellid species correspond to the establishment of one or more adventive species (Harmon et al. 2007) and while a conclusive explanation for the observed declines remains elusive, adventive species have been widely implicated (Obrycki et al. 2000; Evans et al. 2011; Losey et al. 2012a). Twenty-six of the more than 500 coccinellid species currently extant in North America were introduced for biological control (Hodek et al. 2012), the most successful and widespread being *Propylea quatuordecimpunctata*, *Coccinella septempunctata*, and *Harmonia axyridis* which became established in 1972, 1973, and 1988, respectively (Angalet & Jacques 1975; Schaefer et al. 1987; Gordon & Vandenberg 1991). Several factors have been posited as explanations for the apparent

competitive advantage of *C. septempunctata* and *H. axyridis* over native species including that the introduced species are more voracious predators (Cottrell & Yeargan 1998; Yasuda et al. 2004; Finlayson et al. 2010; Hoki et al. 2014) eurytopic, and euryphagous (Hodek & Hoňek 1996; Evans & Toler 2007; Hodek & Michaud 2008) ecologically and phenotypically more plastic (Grill et al. 1997; Labrie et al. 2008), require shorter developmental times (Labrie et al. 2006; Ugine & Losey 2014), and are more fecund (Kajita & Evans 2010) than their native competitors.

These factors could also drive greater effectiveness as biological control agents. Thus the establishment of these two species in particular may, on the surface, appear to be an economically beneficial shift. However, if they displace more than one native species, the resulting decline in coccinellid biodiversity could be detrimental because optimal pest suppression occurs with a diverse assemblage of coccinellids that includes native species (Snyder 2009) and other natural enemies (Gardiner & Landis 2007; Chacon et al. 2008). Some evidence suggests that the addition of introduced species, regardless of relative voracity, may have no net effect on the control of aphids (Evans 1991; Lucas et al. 2002) or even reduce pest suppression (Kajita et al. 2006; Gardiner & Landis 2007; Roy et al. 2008).

The apparent association of native coccinellid decline and the establishment of adventive species in North America has been well documented (Hoebeke & Wheeler 1980; Wheeler & Hoebeke 1995; Brown & Miller 1998; Colunga-Garcia & Gage 1998; Michaud 2002; Alyokhin & Sewell 2004; Evans 2004; Harmon et al. 2007; Finlayson et al. 2008; Hesler & Kieckhefer 2008; Hesler et al. 2009; Losey et al. 2014) and the establishment of *Coccinella septempunctata*, in particular, has been implicated in the decline of several native species including *Adalia bipunctata*, *Coccinella novemnotata*, *Coccinella transversoguttata richardsoni*, *Coccinella trifasciata perplexa, Hippodamia convergens*, and *Hippodamia parenthesis* (Wheeler &

Hoebeke 1995; Elliot et al. 1996; Turnock et al. 2003; Snyder et al. 2004; Harmon et al. 2007; Hoki et al. 2014). In the United States, surveys over the past 20 years and widespread sampling through the citizen-science program The Lost Ladybug Project (LLP 2009) have confirmed the co-occurrence of *C. septempunctata* with the native congeners *C. novemnotata* and *C. transversoguttata* mostly in the Western United States where sizeable populations of the two native species still exist (Losey et al. 2012a). In other regions, especially the Eastern U.S., *C. septempunctata* is now one of the most abundant coccinellids and *C. novemnotata* and *C. transversoguttata*, once common, are either completely absent (e.g., Hesler & Kieckhefer 2008) or present in extremely low numbers (e.g., Losey et al. 2014). It had been more than 14 years since the last *C. novemnotata* individual had been collected in eastern North America when in 2006 a citizen-scientist in Arlington, Virginia found one on their property (Losey et al. 2007).

Various hypotheses have been proposed to explain the rapid and widespread decline of native coccinellids. It is likely that multiple factors are involved, including abiotic influences such as changes in landscape composition (Gardiner et al. 2009), but most research has predominantly focused on antagonistic interactions between introduced and native species. There is a substantial amount of evidence from numerous studies showing that exotic coccinellids impose severe fitness costs on native lady beetles through exploitative competition (Losey et al. 2012b; Hoki et al. 2014), habitat displacement (Evans 2000, 2004, 2010), interference competition through intraguild predation (Elliot et al. 1996; Cottrell & Yeargan 1998; Obrycki et al. 1998; Snyder et al. 2004; Gardiner et al. 2011; Turnipseed et al. 2014; Tumminello et al. 2015; but see Gagnon et al. 2011 and Smith & Gardiner 2013) and the introduction of novel pathogens (Vilcinskas et al. 2013). It is also possible that non-native coccinellids may facilitate the spread of sexually transmitted parasites such as the ectoparasitic fungus *Hesperomyces virescens*, endoparasitic allantonematid nematodes from the genus

Parasitylenchus, and the podapolipid mite *Coccipolipus hippodamiae* (Ceryngier & Twardowska 2013). However, research on the interspecific transmission of parasites, particularly *H. virescens* indicates that while it can and does occur due to the propensity of males of some entomophagous coccinellids to repeatedly initiate copulation with con- and heterospecific males and females (Riddick et al. 2009), it is much less frequent than intraspecific transmission and this is likely due to host-specificity (e.g., Cottrell & Riddick 2012).

Another hypothesis that has been proposed, but not yet tested, is hybridization with introduced species (Evans et al. 2011). Hybridization with an introduced species can have devastating consequences on a native species and under certain conditions is predicted to lead to rapid displacement (Huxel 1999). The severe consequence of hybridization with an introduced species to native species has been documented in a variety of organisms, particularly plants, and in some cases appears to stimulate the evolution of invasiveness (reviewed in Schierenbeck & Ellstrand 2009). Hesler et al. (2010) suggested that perhaps introgressive hybridization could be responsible for some of the variation in elytral macular forms of *Coccinella septempunctata* in areas where it is found with other *Coccinella* species. Careful inspection of certain *C. septempunctata* specimens is sometimes required to avoid mistakenly identifying them as *C. novemnotata* (Hesler et al. 2010) although there are several more reliable characters than elytral maculae that can be used to identify *C. novemnotata* (Brown 1962; Stellwag & Losey 2014). While this variation makes the number of elytral spots a more ambiguous character for separation of *C. novemnotata* and *C. septempunctata*, it is even easier to confuse *C. septempunctata* and *C. transversoguttata* than either species to *C. novemnotata* (personal observation) due to several variations of *C. transversoguttata* in the western U.S. showing a disintegration of the transverse elytral bands into 7, 9, or 11 distinct spots (Figure 1; Dobzhansky 1933). Variation in elytral pigmentation is known to be heritable (Majerus 1994) and thus, it was

reasonable to question the role of hybridization in the appearance of some specimens.

The research presented in this dissertation sought first to determine if hybridization between the introduced species *C. septempunctata* and the native species *C. novemnotata* is possible. When I first started working with coccinellids I encountered the challenge of sexing live adults, a critical skill for doing assays of mating behavior! Thus, my very first objective was to determine reliable methods to differentiate the sexes of species within the *Coccinella* genus with an emphasis on my focal species. The product of this inquiry is the second chapter in this volume in which I describe several very useful sexually dimorphic external characters and the first report of significant sexual size dimorphism in four *Coccinella* species. Additionally, I reviewed the literature and created a table that combines all known sexually dimorphic external traits for North American *Coccinella* species including *C. septempunctata*. The third chapter addresses reproductive interference through interspecific mating between *C. septempunctata* and *C. novemnotata*. Through the first experiment in this chapter I was able to determine that hybridization is not possible between the two species and thus, cannot be contributing to the decline of *C. novemnotata*. However, in a second experiment I quantified significant fitness costs imposed on *C. novemnotata* as a consequence of not only mating with *C. septempunctata* but also as a consequence of prolonged non-sexual interactions. In chapter three I introduce *C. transversoguttata* and use the evolutionary relationships it has with *C. septempunctata* and *C. novemnotata* as a way to test a fundamental hypothesis about the evolution of reproductive isolation. I show that all three species will intermate occasionally, but it is the two species that are allopatric (*C. septempunctata* and *C. novemnotata*) that are most likely to do so. Furthermore, I demonstrate that *C. septempunctata* and *C. transversoguttata* also do not hybridize, but sperm transfer and larval development occurs, the first report of its kind.

As a native species declines and the invasive species becomes more abundant, the

probability that a rare native individual will encounter the more common species increases. If there are substantial fitness costs associated with these interactions, the rare native will continue to decline and possible go extinct. The results of my experiments show that there is a direct fitness cost to a native species through reproductive interference with an introduced species thus shedding additional light on potential mechanisms driving the decline of *C. novemnotata* and other native coccinellids. These insights begin to fill a gap in the published literature and will help facilitate development of effective conservation programs for native coccinellids and perhaps other invertebrates. Finally, the results of my last chapter yielded information that can be used to help estimate the potential impacts of accidental or intentional species introductions on native communities by identifying the evolutionary relationships of the interacting species. Research into biological control will benefit from having another criterion for candidate species evaluation.

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CHAPTER 2

SEXUAL DIMORPHISM IN NORTH AMERICAN COCCINELLIDS: SEXING METHODS FOR SPECIES OF *COCCINELLA* L. (COLEOPTERA: COCCINELLIDAE) AND IMPLICATIONS FOR CONSERVATION RESEARCH[1]

ABSTRACT

Coccinellids typically do not show exaggerated sexual dimorphisms and the only reliable sexing methods for some species have been dissection and behavioral observations. Behavioral methods can potentially lead to sex identification but are very time consuming, require exposing sexually naïve individuals to conspecifics, and risk incorrect identification since homosexual mounting in these species has been observed in the laboratory. Research involving use of live specimens requires techniques to non-invasively sex individuals, but such methods have not been clearly or fully described in the literature. Closer examination of the species *Coccinella novemnotata* Herbst*, C. septempunctata* L., *C. transversoguttata richardsoni* Brown, and *C. trifasciata perplexa* Mulsant has led to the discovery of a reliable and efficient way to differentiate the sexes by looking at the shape of the seventh sternite (fifth visible) and has been demonstrated to be 100% reliable for all four species. Another, even more rapid, method uses the shape of a prominent pronotum marking and shows promise for *C. novemnotata* but is not applicable to the other species. Additionally, most *Coccinella* L. species have males with conspicuous pale anterior coxal spots and femur stripes that can be easily viewed even on mobile specimens.

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In this paper we report on morphometric data that quantify external dimorphisms and provide evidence for the reliability of using them for sexing. All known external characters that can be used for sexing North American *Coccinella* species are consolidated for easy reference. The significance of these findings for research into the decline of native *Coccinella* species in the United States is discussed.

KEY WORDS: *Coccinella*, morphology, native species, pronotum pattern, sternite

INTRODUCTION

The ability to quickly and accurately determine the sex of insects using non-invasive methods is often required for research purposes. *In-situ* sex-determination of live specimens allows for the assessment of a population's sex ratio, which can be used to make inferences about the vulnerability of declining or threatened species (Gilpin and Soulé 1986). In the laboratory, determining the sex of adults using external characters yields sexually naïve individuals that can be used in behavioral assays.

Some beetle species are so sexually dimorphic that females and males may be misidentified as separate species, making sexing easy (e.g., rhinoceros beetles - Dynastinae). Lady beetles (Coleoptera: Coccinellidae) are not characterized by extreme sexual dimorphism and no external characteristics are universal for sexing (Hodek 1973; Gordon 1985; Majerus 1994). While males are usually smaller than females and have slightly longer antennae (Smith 1966; Hodek 1973; Hodek and Honěk 1996) the most reliable sexing method is checking for the presence or absence of a long, curved, sclerotized sipho ("penis" or "aedeagus") that is only visible during copulation or by dissection of the abdomen (Hodek and Honěk 1996; the sipho is more obvious than the relatively small and usually obscured spermatheca in females), however, noninvasive techniques are required if specimens are to be used alive for research, especially when working with rare or declining species (e.g., Losey *et al.* 2007). Although lady beetles are not highly sexually dimorphic, some subtle differences exist. For example, in many species the posterior margin of the eighth sternite (sixth visible – because sternites one and two are fused) is emarginated (i.e., notched) medially in males (Hodek 1973; Hodek and Honěk 1996).

The literature for sexing lady beetles is sparse and species-specific or gives sexing methods that are vague and not described in a way that is useful for other researchers. For example, to sex adults for experimentation, Harmon *et al.* (2008) observed the "ventral aspect of

[the] abdomen" of *Coleomegilla maculata* (De Geer) using a stereomicroscope and confirmed this method to be >95% accurate through dissection, but the abdominal character used is unspecified. In other papers, sexing methods are completely omitted (e.g., Yasuda and Dixon 2002). Detailed morphological descriptions of sexually dimorphic traits in Coccinellids exist only for *Propylea quatuordecimpunctata* L. (Rogers *et al.* 1971), *Coleomegilla maculata* (Nichols and Neel 1974), *Coccinella septempunctata* L. (Baungaard 1980), *Chilocorus nigritus* (F.) (Samways and Tate 1984), *Hippodamia* (*Adonia*) *variegata* (Goeze) (Hurst *et al.* 1999), and *Harmonia axyridis* (Pallus) (McCornack *et al.* 2007). Details regarding the character or characters that can be used reliably for sexing are critical for behavioral studies requiring naïve specimens. Knowing not only which characters can be used for sexing but also their reliability can save time, money and improve research quality.

In several species, the shape of the seventh (McCornack *et al.* 2007) and/or eighth (Baungaard 1980; Samways and Tate 1984) sternite is distinctly sexually dimorphic. Males have a medial emargination or scalloping along the posterior edge (Hodek 1973) and at least in the case of *Harmonia axyridis* this is not dependent upon rearing temperature, diet, or days since eclosion (McCornack *et al.* 2007). Hurst *et al.* (1999) describe a "ventral 'notch' in the posterior margin of the posterior abdominal tergite" in males of *Hippodamia variegata* (it is assumed that the authors meant sternite, the number of which is unspecified) whereas females have a more rounded edge and this character was verified through dissection to be 100% accurate for sexing.

Another character that can be sexually dimorphic in Coccinellids is the presence, absence, or pattern of pigments; in some species males have lighter or smaller area of dark pigmentation on the head or other body parts (Hodek 1973). In *Propylea quatuordecimpunctata* the degree of black pigmentation on the head is sexually dimorphic with females having a distinct black pattern that is lacking, but sometimes ambiguous or unreliable, in males (Rogers *et*

al. 1971). A more obvious, but less reliable, character is the pattern of pigmentation on the pronotum. *P. quatuordecimpunctata* females also have a greater proportion of black pigmentation on the pronotum and males have a distinct medial emargination along the anterior edge of the black area whereas females have only a vague emargination (Rogers *et al.* 1971). Additionally, this species has a sexually dimorphic pattern of pigmentation on the prosternum and prosternal carina with females being darker (personal observation). A recent test of the black patterns of the three areas: head, prosternum, and prosternal carina revealed that they are 81, 94, and 71 percent reliable (respectively) in differentiating the sexes in *P. quatuordecimpunctata* (L. Stellwag, unpublished data). *Hippodamia variegata* (Goeze) also shows a sexually dimorphic pattern to head and pronotum patterns, again with females having a larger area of black pigmentation on the head and pronotum (Gordon 1987). While pigment pattern is usually conveniently detectable by the unaided eye, it can be influenced by environmental conditions (e.g., rearing temperature and diet), days since eclosion (McCornack *et al.* 2007), appears to be sexually dimorphic in only a few species, and is sometimes ambiguous. Given the frequent ambiguity of pronotal and/or head pigment pattern, the shape of one or more posterior sternites appears to be the most reliable and convenient character to use for sexing live Coccinellids, but a quantitative evaluation across multiple species has never been performed.

Several species of North American lady beetles, in particular *C. novemnotata* Herbst, *C. transversoguttata richardsoni* Brown, and *Coccinella trifasciata perplexa* Mulsant have declined over the past 20-30 years (Turnock *et al.* 2003; Harmon *et al.* 2007). While the decrease in abundance of these species corresponds with the establishment of the exotic species, *Coccinella septempunctata* (Wheeler and Hoebeke 1995) the exact mechanisms are still unknown. In order to test hypotheses about the potential causes and consequences of native lady beetle decline, a quick, reliable, and noninvasive sexing technique is needed. No obvious external characteristics

have been found to be consistent for all *Coccinella* L. species (Brown 1962). However, the eighth abdominal sternite is sexually dimorphic in the adventive *C. septempunctata* (Baungaard 1980) and it has been observed that the shape of the dark pronotum markings of *C. novemnotata* appears to be sexually dimorphic, but this has never been confirmed. In at least half of the native North American *Coccinella* species males have a large white spot on the anterior face of each anterior coxa and a white stripe on the ventral face of each anterior femur (Brown 1962) and these are easily visible on mobile specimens without using magnification. Understanding which externally visible characters can be used for sexing and, more importantly, their reliability has broad applications in Coccinellid research. A synthesis of information on sexing Coccinellids, especially genera containing species of conservation interest, is currently lacking.

The objectives of the present study were to 1) review the literature to identify which external characters have been described for differentiating females and males for all native North American *Coccinella* species and *C. septempunctata*; 2) determine if one of the posterior abdominal sternites is significantly sexually dimorphic through quantitative analysis in three native species: *C. trifasciata perplexa*, *C. transversoguttata richardsoni*, and *C. novemnotata*, and the adventive *C. septempunctata*; 3) evaluate the accuracy of using an abdominal sternite to differentiate the sexes for as many North American *Coccinella* species as possible (using pinned specimens when live beetles were not available); 4) describe and quantify the sexually dimorphic pronotum markings in *C. novemnotata* and to test the reliability of using this character to sex live specimens; and finally, 5) pull together disparate information on methods of sexing North American Coccinellids, in particular *Coccinella* species, into a single, easily-accessible resource to facilitate future research.

MATERIAL AND METHODS

Specimens. All specimens used in quantitative analyses originated from lab-reared colonies that were started with field-collected individuals from Colorado, Oregon, and South Dakota in 2009 and Oregon in 2012. Specimens for qualitative analysis were obtained from the museum collections at Cornell University, University of California-Davis, and McGill University (pinned); live *Coccinella monticola* Mulsant specimens were collected from Colorado in 2013.

Quantitative Analyses

Sexual size dimorphism. To determine the degree, if any, of sexual size dimorphism in *C. trifasciata perplexa* (C3), *C. transversoguttata richardsoni* (CT), *C. novemnotata* (C9) and *C. septempunctata* (C7), body measurements to the nearest one hundredth of a millimeter were taken using digital calipers (n=40, 20 of each sex for each species). Length (L) was used as an indicator of overall body size and was measured from the anterior edge of the head to the posterior medial tip of the elytra.

Sternite shape and pattern of pronotum pigmentation. Photographs of recently freshly killed specimens were taken using an Olympus DP25 digital camera mounted to a dissecting microscope with Olympus Micro Image Analysis Software (DP2-BSW). Dimensions of the seventh abdominal sternite (S7, fifth visible; all species) and anterior margin of the pronotum pigmentation (C9 only) were measured using Adobe Photoshop CS4 Extended Version 11.0. A reference standard was generated for each image and the distance between two points were measured to the nearest one one-thousandth of a millimeter.

Five measurements of S7 were taken for C3, CT, C9 and C7 (n=40, 20 of each sex for each species). Width was determined by measuring the length of the anterior (wa;

Figure 1) and posterior edges (wp). Length was measured at three points (hm, hl, and hr): the distance between the end of the sixth sternite and the beginning of the eighth at the midline (hm) and lateral posterior endpoint of S7 at the left and right edges (hl, hr) along a line that ran parallel to the midline. To account for asymmetry, the lengths at the endpoints (hl and hr) were averaged. Hm was divided by this average to generate a proportion (hm-prop) that describes the degree of emargination at the midline and approaches 1.0 as uniformity increases.

Two measurements were taken for the shape of the pronotum pigmentation in C9 (n=20 per sex), the width at the widest anterior points (p-width) and the distance from the pigment edge at the midline to a line drawn to connect the two endpoints from the width measurement (p-depth; Figure 2). Depth was divided by width to yield a standardized measure of divergence of the pigment edge from the midline (p-prop) that controlled for variation in overall body size.

Statistical analyses for morphological measurements. The black pronotum pattern for C9 (p-width, p-depth, and p-prop) and body length (L) and sternite measurements wa, wp, hm, and hm-prop for all species were tested for sexual dimorphism using receiver operating characteristic (ROC) analyses in JMP Pro (Version 10. SAS Institute Inc., Cary, NC, 1989-2010) and SPSS (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) statistical software packages. The true state was set as female for all analyses except for p-depth (C9) and wp (C3).

ROC analyses yield the probability of making true and false positive as well as true and false negative diagnoses and are used in a variety of fields including weather forecasting, aptitude testing, medical imaging, and polygraph lie detection (Swets 1988). Collected data values are used to generate a ROC curve that graphically depicts the true positive probability, which is reported as the area under the ROC curve (abbreviated

AUC; Fawcett 2006). If no discrimination exists (true and false positive proportions are equal) the AUC will be 0.50. The more reliable a character is for generating a true positive diagnosis, in this case the probability of correctly identifying a female specimen, the higher the AUC (maximum = 1.0). For each measurement a range of "threshold values" is produced with an associated degree of accuracy. Thus, for each character in the present study, the AUC, standard error, 95% confidence interval, p-value, and threshold value that maximizes true positive while minimizing false positive probabilities was reported.

Blind sexing tests for *Coccinella novemnotata.* Reliability and accuracy of sexing C9 using S7 shape, black pronotum pattern, and anterior coxal spots and femur stripes was conducted using independent blind tests of live specimens (n=40, 20 per sex). The correct sex of each specimen was determined in advance using behavioral observations, beetles that had not laid eggs were paired with ones that had until copulation was observed. Beetles were kept individually in 44ml plastic cups, given an arbitrary number, and identified as male or female based only on the character being examined and without knowledge of the correct sex. If the character was ambiguous, a best "guess" was made. A specimen was identified as male if its S7 had a distinct emargination at the midline, possessed pale coxal spots and femur stripes on the anterior legs (Figure 3), or, in the case of C9, had an inverse peak in the anterior margin of the black pronotal marking (e.g., Figure 2). During examination, beetles were restrained using a plastic petri dish filled with cotton such that when the lid was applied the beetle was immobilized with its venter clearly visible. Two researchers using the same specimens to compare accuracy conducted the tests for all three characters independently. A successful identification was considered only for specimens that were correctly sexed by both researchers. The percentage of successful identifications for each of the three

tests was analyzed in JMP Pro 10 using binomial exact tests with an expected chance probability of making a correct identification equal to 0.5.

Qualitative Analyses

Pinned and field-collected specimens. Pinned museum specimens were used to determine if S7 is sexually dimorphic for the following species: *C. californica* Mannerheim (n=20), *C. johnsoni* Casey (n=3), *C. prolongata bridwelli* Nunenmacher (n=3), *C. difficilis* Crotch (n=11), *C. undecimpunctata undecimpunctata* L. (n=22), and *C. hieroglyphica kirbyi* Crotch (n=15). Because sex could not be confirmed via dissection or behavior it was assumed that if two distinct sternite morphotypes were observed, it is sexually dimorphic. If only one morphotype was observed, it was assumed that all specimens were either of the same sex or the sternite is not sexually dimorphic thus, no conclusion could be made. In species for which males are known to always have pale coxal spots (*C. californica, C. johnsoni*, *C. difficilis*; Brown 1962; Figure 3) their presence was used as confirmation that a specimen was male. Likewise, the absence of coxal spots in these species was confirmation of female sex. The absence of coxal spots in *C. prolongata bridwelli, C. undecimpunctata undecimpunctata,* and *C. hieroglyphica kirbyi* was not used as an indicator for sex because in these species males may have obscure spots or lack them altogether (Brown 1962). For all species, if the position of the specimen's legs prevented adequate viewing of the coxae, sex could not be confirmed using coxal spots.

F1 progeny from a *Coccinella monticola* colony were examined for S7 morphology and sex was confirmed via dissection (n = 10 per sex). Twenty (10 per sex) laboratory-reared *C. septempunctata* were examined to determine if pronotum and head patterns are sexually dimorphic in that species and if males posses pale coxal spots or femur stripes.

Sexing *Coccinella novemnotata* using pronotum pattern in photographs. The citizen science program, The Lost Ladybug Project (LLP) has received over 22,000 images of lady beetles since 2008 and a method of determining the sex of specimens in photographs would be valuable in assessing sex ratios of declining native species. Because photographs almost always show only the dorsum, ventral characters are not visible. To assess the viability of using the pattern of pronotum pigmentation in C9 for sexing we accessed all photographs of C9's submitted to the LLP website (www.lostladybug.org) on 20 June, 2013. Beetles were sexed according to the same criterion used in the blind test for pronotum pattern. Only beetles for which the pronotum was clearly visible were evaluated. One hundred sixty-three photographs depicting a total of 171 specimens were used.

RESULTS

Quantitative analyses

Body length and dimorphism of the seventh sternite. All species showed sexual size dimorphism with females significantly larger than males and most measurements of S7 were significantly sexually dimorphic across species (Table 1)**.** ROC analyses revealed that using hm-prop yielded correct sex discrimination 100% of the time for all four species (Table 2). Figure 1 provides a photographic comparison of S7 for a male and female C9. Body length, while significantly different between the sexes across species, was the least reliable character (Table 2).

Pronotum pattern in *Coccinella novemnotata.* The two measurements of the pronotum pattern in C9 were highly sexually dimorphic (Table 1). Males had an inversed peak at the midline of the black pronotum markings that was three times deeper than females (mean p-depth for females = 0.110 mm, SD = 0.05 versus mean of 0.33 mm for

males, SD = 0.08). P-depth was significantly accurate at predicting sex with an AUC = 0.998 ($p < 0.01$). P-prop was 97.5% reliable (Table 2) in predicting sex.

Blind sexing tests for *Coccinella novemnotata.* Using pronotum pattern to visually sex C9 specimens yielded successful identification 67.5% of the time with males much more likely to be incorrectly labeled female (12 males and one female misidentified) and this was significantly greater than the expected chance probability ($p = 0.019$). Both researchers arrived at the same incorrect identification for all but one male specimen. Using coxal spots and the shape of S7 always yielded correct identifications.

Qualitative analyses

Sexually dimorphic character evaluation for North American *Coccinella* species. Examination of pinned museum specimens revealed two morphotypes of S7 in *C. californica, C. johnsoni,* and *C. prolongata bridwelli.* In *C. difficilis* only one morphotype was observed in 3 specimens. Only one morphotype was evident in *C. undecimpunctata undecimpunctata* and *C. hieroglyphica kirbyi* however, the eighth sternite was dimorphic in both species. Table 3 displays this information as well as the applicability of all known sexually dimorphic characters for North American *Coccinella* species and *Coccinella septempunctata* as determined by the present study and the published literature. Sex of males was confirmed in several specimens based on the presence of pale coxal spots. The following sex identifications were made based on sternite shape in combination with the presence or absence of coxal spots for presumed males: *C. californica* (13 F; 7 M - all with coxal spots), *C. johnsoni* (1 F; 2 M - both with coxal spots), *C. prolongata bridwelli* (1 F; 2 M - both with coxal spots), *C. difficilis* (8 F; 3 M - all with coxal spots), *C. undecimpunctata undecimpunctata* (12 F; 10 M - 5 with distinct coxal spots, 2 lacking spots, 3 with vague, brown coxal spots), and *C. hieroglyphica kirbyi* (7 F; 8 M - all with spots). Examination of *C. monticola* specimens

revealed two S7 morphotypes but the emargination in males was much less pronounced than in other species. *C. septempunctata* males never had coxal spots or femur stripes and pronotal and head patterns were as described in Gordon (1985) with no distinction between the sexes.

Sexing *Coccinella novemnotata* in online photographs. Of the 171 C9 specimens examined in online photographs, 38 had obscured views of the pronotum and could not be sexed, 86 were identified as female, and 47 as male.

DISCUSSION

A review of the literature for determining sex within the genus *Coccinella* yielded little information beyond descriptions of internal genitalia (e.g., Baungaard 1980). However, Brown (1962) indicated that males of several *Coccinella* species always have anterior pale coxal spots and femur stripes (anterior legs only; Figure 3), but in some species the spots may be obscured or stripes absence. Of the 13 species examined in the present study, all except *C. fulgida* Watson, *C. monticola* and *C. septempunctata* had males with coxal spots at least some of the time and 5 of the 10 species with coxal spots had femur stripes. When present, pale coxal spots and femur stripes are obvious to the unaided eye and are thus suitable for quick and easy sexing in species in which males *always* possess them. Caution must be exercised when using this character for species in which males sometimes lack the spots, have ones that are difficult to see (e.g., *C. hieroglyphica kirbyi*), or when using old specimens for which the spots may have faded and sex should be confirmed with another character known to be consistently sexually dimorphic regardless of environmental conditions (e.g., shape of S7). Table 3 provides a consolidated account of known external sexing characters for *Coccinella* species. Such a resource is needed since many characters are scattered among disparate sources. For

example, Brown (1962) described the pale coxal spots and femur stripes but this was completely omitted from the seminal work by Gordon (1985). Having this information and other published details in a centralized location is a useful tool for future research.

Sexual dimorphism in abdominal sternites. In male Coccinellids, the last abdominal sternites must be shaped in such a way as to permit extension and proper positioning of the sipho for copulation. A posterior emargination of the last sternites allows forward bending of the abdomen for intromission. In the present study, the seventh abdominal sternite in *Coccinella trifasciata perplexa*, *C. transversogutta*, *C. novemnotata*, and *C. septempunctata* was determined to be highly sexually dimorphic, with females lacking a medial emargination, and assessing it visually was 100% reliable as a sexing method. This sternite was found to be also sexually dimorphic in several other species within the genus through examination of museum specimens (Table 3). In at least two species, S7 appeared monomorphic but the eighth sternite (S8) was dimorphic. Of the 12 native *Coccinella* species (not counting subspecies separately), ten were tested, eight confirmed to have a sexually dimorphic S7, and the two species lacking this characteristic had a sexually dimorphic S8. Assuming all subspecies have the same sternite characteristics [this was true for the subspecies *C. trifasciata perplexa* and *subversa* (LeConte)], only two species remain unconfirmed: *C. alta* Brown and *C. fulgida*.

Using the shape of S7 to sex Coccinellids is convenient and preferred to using S8 because S7 is much larger and consequently, more obvious. Baungaard (1980) reported using S8 in *C. septempunctata* and included a description of the tuft of hairs that occurs in the concave portion of S8 (males only), a feature that is immediately apparent when viewed under magnification. The tuft of hairs is as conspicuous as S7 shape and is the easiest way to sex this species (personal observation). When examining both *C.*

trifasciata subspecies we noticed a similar feature- males have a greater density of hairs lining the posterior edges of S7 and S8 and this characteristic makes males easier to identify than only using S7 shape.

Even though the dimorphic shape of the last abdominal sternites is obvious to the trained eye, magnification and beetle immobilization is required, making in-field sex determination challenging. Other limitations include ambiguities in sternite shape in some species. In *Harmonia axyridis*, the last two abdominal sternites are sexually dimorphic (Riddick and Schaefer 2005) and similarly, S7 is easier to view than S8. However, in females, S7 is convex at the midline and sometimes transparent making it difficult to see. Consequently, frequent misidentification of females is possible (McCornack *et al.* 2007). In *Coleomegilla maculata* males have a very slight medial emargination in the last visible sternite and this trait has been used successfully for years for sexing (J. Lundgren, pers. comm.) although to the untrained eye the difference is small and frequently ambiguous (personal observation). Despite the limitations in some contexts, in contrast to other methods, such as squeezing the abdomen of live specimens with forceps until the genitalia protrude (Nichols and Neel 1974) sexing using sternite shape is quick, harmless, accurate, and reliable, making it an easy and effective technique. That S7 is unambiguously distinct between males and females for the *Coccinella* species examined herein is fortuitous.

Pronotum pattern in *Coccinella novemnotata.* Our study corroborates previous work (e.g. Rogers *et al.* 1971), by determining that for *Coccinella novemnotata*, pronotum pattern is frequently sexually dimorphic however, it is prone to substantial ambiguity. This is the first study to investigate the utility of using pronotum pattern for sexing C9 and the first report of this type of sexual dimorphism within the *Coccinella* genus. While males typically had a deeper emargination at the midline (p-depth; Figure

2), there was considerable variation, leading to a higher occurrence of males being misidentified as females. Using pronotum pigmentation allowed for correct sex determination at a rate (67.5%) significantly higher than would be expected by chance (50%) but this does not seem sufficient for sex-ratio research. Because males were more likely to be misidentified as female, if pronotum pattern is the only characteristic used for sexing, the number of females will likely be inflated and males underestimated. As a qualitative sexing method, we conclude that C9 pronotum pattern should only be used when the venter is not accessible and recommend using caution when drawing conclusions about sex ratio if using this character alone. Sexing C9 specimens in online photographs from the Lost Ladybug Project using pronotum pattern alone revealed that over 20% of the specimens had obscured views of the pronotum and could not be sexed, 50% were assumed to be female, and 27% male. Given the success rate of 67.5% in the blind test these numbers are likely skewed due to misidentification of males as female. Unfortunately, there was no way to confirm the identity of specimens so this result is of limited utility.

As a quantitative measure, the shape of the pronotum pattern via the calculation p-prop (Figure 2) was very reliable, leading to only one false negative (a female incorrectly labeled as male). This high degree of accuracy means that there is a pronounced distinction between males and females, however, getting such an accurate result requires detailed measurement and quantitative analysis and may be too time consuming or impractical for most studies. The most accurate method of sexing beetles in online photographs would be to take measurements and calculate p-prop, but variation in magnification and angle would likely confound the results. Therefore, knowing that p-prop is significantly different between the sexes in C9 is interesting but the practical applications have yet to be discovered.

Sexual size dimorphism. In at least 29 species of Coccinellids males are smaller than females (Dixon 2000). In a study of the effect of diet using 13 aphid species, female *Coccinella septempunctata* weighed significantly more than males regardless of aphid species (Kalushkov and Hodek 2004). One possible explanation for the female bias in sexual size dimorphism in Coccinellids as well as other holometabolous insects is that males begin gonadal development before females and the gonads compete with somatic cells for resources that could be allocated to somatic cell growth (Yasuda and Dixon 2002; Dixon 2000). Additionally, females may require greater body mass simply because eggs are several orders of magnitude larger than sperm. The results of the present study are consistent with previous findings in that the four species *C. trifasciata perplexa*, *C. transversogutta richardoni*, *C. novemnotata*, and *C. septempunctata* showed significant female-biased sexual size dimorphism. However, using size, quantified by body length, as a predictor of sex always lead to multiple false positives and negatives (Table 2) indicating that the size difference between males and females is too small and variation too high for size to be a reliable *qualitative* predictor of sex.

Comparison of potential sexing characters for lady beetles in the *Coccinella* genus. Based on our analysis of several sexually dimorphic external characters within the genus *Coccinella*, we conclude that dimorphism of the abdominal sternites, usually the seventh (fifth visible), but sometimes the eighth, is the most ubiquitous and reliable non-invasive sexing technique. While other characters, such as pronotal pattern and coxal spots are useful for the species in which they are *reliably* sexually dimorphic, dimorphism in the abdominal sternites provides the highest accuracy and the broadest applicability. Our data supports the use of this character in situations where restraining the beetle is feasible. Interestingly, C3, CT, and C9 have males with anterior coxal spots and femur stripes while C7 does not and C9 is the only species of the four with a

dimorphic pronotum pattern. Understanding the distribution of these characters within the genus may be helpful in resolving phylogenies. Further studies on similar characteristics, measurements and indices that include species that were unavailable at the time of this study could lead to even more effective sexing methods.

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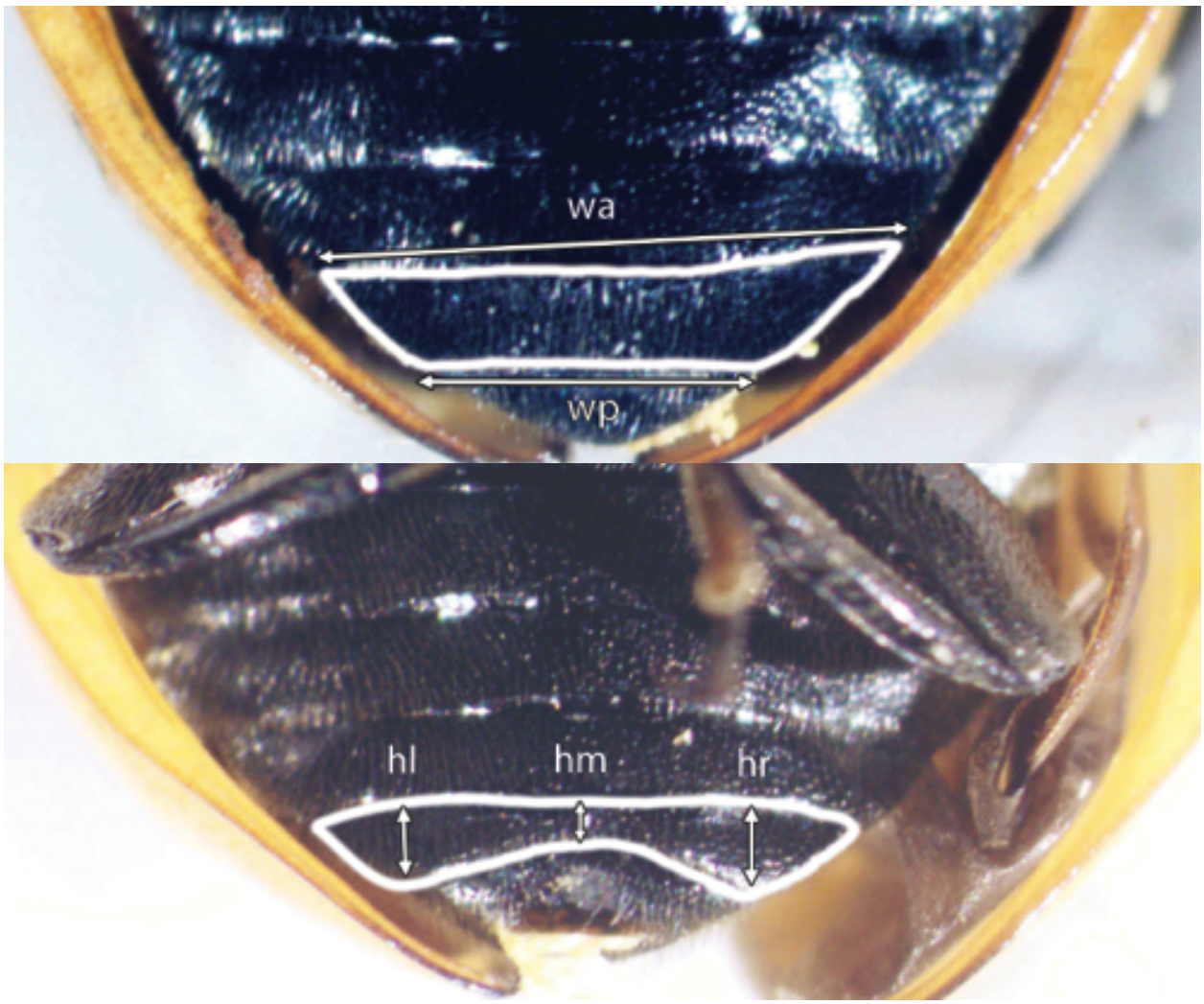

Figure 1: Dorsum of a *Coccinella novemnotata* female (top) and male showing the dimorphic seventh abdominal sternite (S7; outlined) and the location of the five measurements taken from all specimens. Note the emargination at the midline in the male. This constriction was estimated using hm-prop (hm divided by the average of hl and hr). Photos by L. Stellwag.

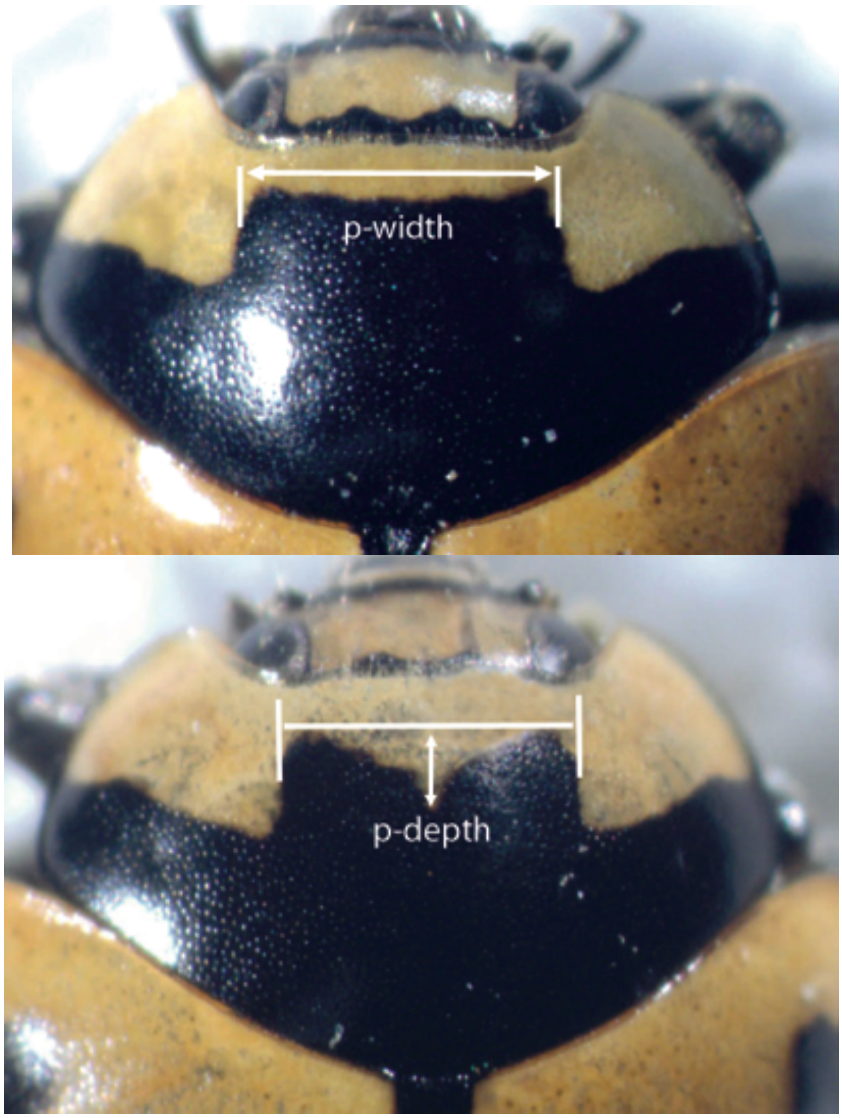

Figure 2: Pronotum pattern on a *Coccinella novemnotata* female (top) and male (bottom). Two measurements were taken: the width at the widest anterior points (p-width) and the depth from the pigment edge at the midline to a line drawn to connect the two endpoints from the width measurement (p-depth). To control for size differences between males and females p-depth was divided by p-width and this proportion was used for analysis (p-prop). Photos by L. Stellwag.

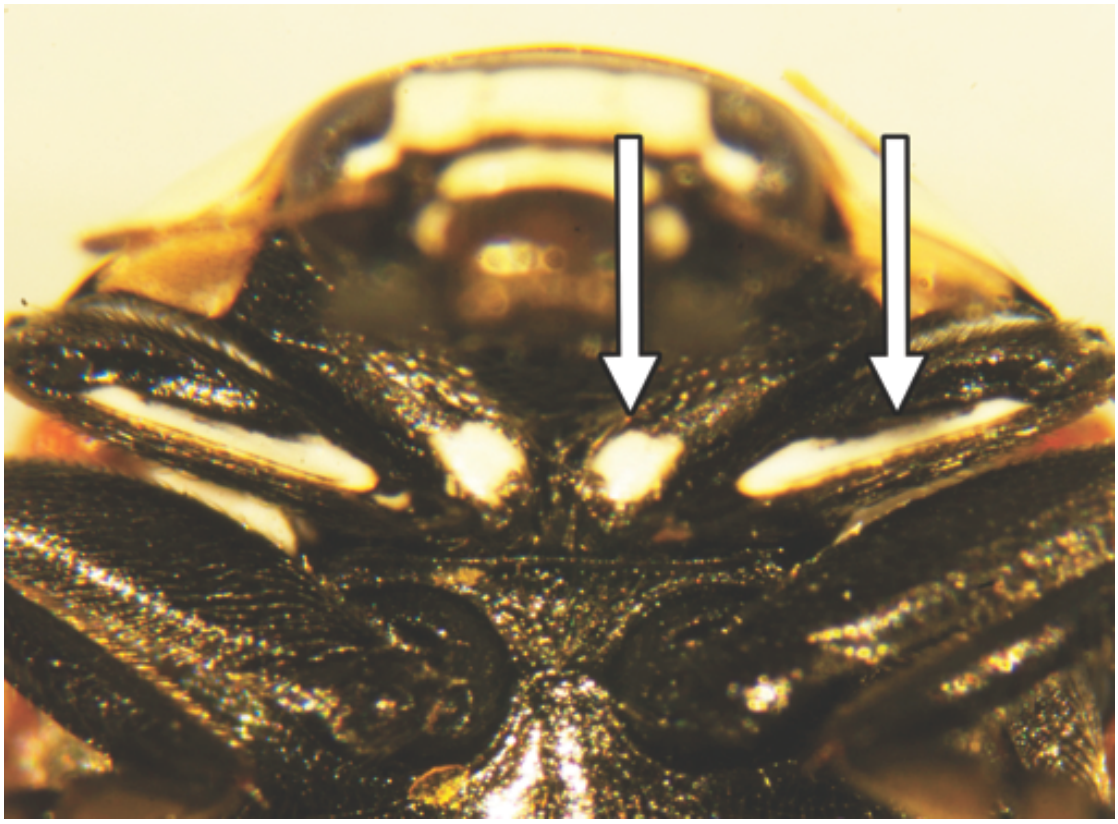

Figure 3: *Coccinella novemnotata* male showing the white spot on the anterior face of the anterior coxa (left arrow) and white stripe on the ventral face of the anterior femur. Females always lack and males of this species always have these characters, which are visible with the unaided eye. See Table 3 for occurrence of this character in other North American *Coccinella* species. Photo by Todd A. Ugine.

Table 1: Results of ROC analyses for sexual dimorphism in body length, five measurements of the seventh abdominal sternite (wa, wp, hl, hm, hr) for *Coccinella trifasciata perplexa* (C3), *C. transversoguttata richardsoni* (CT), *C. novemnotata* (C9), and *C. septempunctata* (C7) and two measurements for pronotum pigmentation in C9. See Figures 1 and 2 for character explanations.
*p < 0.05 **p < 0.001 ns = not significant

	Species			
Character	C3	CT	C9	C7
length	**	**	**	**
wa	*	*	ns	*
wp	ns	ns	ns	ns
hl	*	**	*	**
hm	**	**	**	**
hr	*	**	*	**
p-width	n/a	n/a	**	n/a
p-depth	n/a	n/a	**	n/a

Table 2: Summary statistics and results of ROC analyses for hm-prop and body length (all species) and p-prop (C9 only). AUC is the area under the ROC curve; the standard error (SE) and 95% confidence intervals are for the AUC. The threshold (Thr) is the value for the character at or above which the probability of correct identification is maximized. Reliability (Rel) is n (40 for all) minus error (false negative + false positive) divided by n. The true state was female for all species thus a false positive occurred when a male was incorrectly identified as female and a false negative was a female that was incorrectly identified as male based on the threshold value.

Species	Character	Mean (SD) Female	Mean (SD) Male	AUC	SE	95% Lower	95% Upper	Thr	Rel%	False pos-neg	p
C3	HM-prop	1.05 (0.06)	0.57 (0.20)	1.0	0	1.0	1.0	0.929	100	0-0	0
	Body length	5.25 (0.21)	4.79 (0.25)	0.952	0.029	0.895	1.0	5.090	87.5	1-4	0
CT	HM-prop	1.02 (0.07)	0.73 (0.06)	1.0	0	1.0	1.0	0.883	100	0-0	0
	Body length	6.36 (0.57)	6.01 (0.32)	0.762	0.079	0.607	0.918	6.370	77.5	2-7	0.005
C9	HM-prop	0.95 (0.05)	0.65 (0.10)	1.0	0	1.0	1.0	0.864	100	0-0	0
	Body length	6.17 (0.28)	5.69 (0.29)	0.876	0.057	0.765	0.987	6.010	87.5	1-4	0
	P-Prop	0.07 (0.03)	0.21 (0.05)	0.995	0.007	0.982	1.0	0.112	97.5	0-1	0
C7	HM-prop	1.06 (0.08)	0.71 (0.06)	1.0	0	1.0	1.0	0.940	100	0-0	0
	Body length	6.84 (0.51)	6.30 (0.55)	0.785	0.076	0.636	0.934	6.680	77.5	4-5	0.002

Table 3: Summary of sexually dimorphic traits in North American *Coccinella* species (including *C. septempunctata*) as determined by a review of the literature and findings of the present study. Previously published information for native species is from Brown (1962); information for *C. septempunctata* is from Baungaard (1980). Native species are arranged phylogenetically according to Brown (1962). The coxal spot is on the anterior face of each anterior coxa only. The femur stripe is on the ventral face of each anterior femur. No spots or stripes occur on the other legs. S7 is the seventh abdominal sternite (fifth visible). Symbols: + (present), − (absent), ∅ (unverified/unpublished), **bold** = present study, S8 = eighth sternite sexually dimorphic

Table 3:

Coccinella species	Anterior coxal spot (male)	Anterior femur stripe (male)	Dimorphic pronotum pattern	Dimorphic head pattern	Dimorphic S7
trifasciata perplexa	+	–	a	b	+
trifasciata subversa	+	–	–	c	$+_{\mathbf{d}}$
transversoguttata richardsoni	+	+	–	–	+
transversoguttata ephippiata	+	+	–	–	∅
californica (n=20)	+	+	–	–	$+_{\mathbf{e,f}}$
johnsoni (n=3)	+	+	–	–	$+_{\mathbf{e,f}}$
novemnotata	+	+	**g**	–	+
prolongata prolongata	+	h	–	–	∅
prolongata sequoiae	+	–	–	–	∅
prolongata bridwelli (n=3)	i	–	–	–	$+_{\mathbf{e,f}}$
alta	j	–	–	–	∅
difficilis (n=11)	+	+	–	–	$+_{\mathbf{k}}$
fulgida	–	–	–	–	∅
undecimpunctata undecimpunctata (n=22)	l	–	–	–	$\mathbf{S8_{e,m}}$
monticola	–	–	–	–	$+_{\mathbf{d}}$
hieroglyphica kirbyi (n=15)	n	–	–	–	$\mathbf{S8_{e,m}}$
hieroglyphica mannerheimii	n,o	–	–	–	∅
hieroglyphica humboldtiensis	n	–	–	–	∅
septempunctata	$-_{\mathbf{p}}$	$-_{\mathbf{p}}$	$-_{\mathbf{d}}$	$-_{\mathbf{d}}$	$+_{\mathrm{q}}$

Table 3 (Continued):

[a]Pronotum with the anterior margin pale at middle except in some females
[b]Head pale except for a black band across the base (male) or black with two pale spots (female)
[c]Head of the female blackish with a broad, pale band between the eyes; the band rarely very narrowly interrupted medially. Other characters as in *t. perplexa.*
[d]Personal observation of live specimens, measurements not taken
[e]Personal observation of museum specimens
[f]At least one specimen had pale coxal spots (confirming specimen was male) and had emargination of S7 while at least one specimen lacked coxal spots (confirming specimen was female) and lacked emargination of S7
[g]Sometimes ambiguous, described in present study
[h]Ventral face of each anterior femur sometimes with a transverse pale spot near the trochanter
[i]Spots may be absent
[j]Anterior coxae entirely black or with a somewhat pallid area on the anterior face of each
[k]Looked at 3 pinned specimens with emarginated S7 and 8 live specimens that were confirmed to be female (ovipositing) and lacked S7 emargination
[l]Anterior coxae usually entirely black, rarely with a strongly infuscated pale spot on the anterior face of each
[m]Two morphotypes present for S8 but not S7 (personal observation)
[n]Coxal spots usually somewhat obscure
[o]Coxal spots frequently entirely black
[p] First report of the lack of this character
[q]Baungaard (1980) reports that S8 is truncate at apex having a transverse fovea in males

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CHAPTER 3

HYBRIDIZATION AND SATYRIZATION: THE ROLE OF REPRODUCTIVE INTERFERENCE BY *COCCINELLA SEPTEMPUNCTATA* ON THE DECLINE OF *COCCINELLA NOVEMNOTATA* IN NORTH AMERICA

ABSTRACT

To determine if the lady beetles *Coccinella novemnotata* (C9) and *Coccinella septempunctata* (C7) can hybridize, virgin adults were paired with a heterospecific of the opposite sex and allowed to copulate and eggs collected. C9 females that successfully copulated with a C7 male were subsequently mated with a conspecific male. Fecundity and percent hatch was compared pre- and post-conspecific mating to determine if the C7 copulation imposed a fitness cost to the C9 female. In another experiment, C7 and C9 beetles that interacted with a heterospecific 1-6 consecutive times without copulating were given an opposite-sexed conspecific to determine the impact of non-sexual contact with a heterospecific on the propensity to mate with a conspecific. C7/C9 pairs copulated, but did so rarely (19.6%) and while females of both species always produced eggs, none developed. C9 females that mated with a C7 male laid more and cannibalized a lower proportion of eggs after mating with a conspecific. The same females showed a significant reduction in the number of eggs laid and hatched after mating with a conspecific compared to control females that only mated with a conspecific. There was no change in the propensity of C7 individuals to mate conspecifically after repeated non-sexual contact with C9. However, there was a significant decrease (31% lower than control group) in the propensity of C9 individuals to mate with a conspecific as the number of non-sexual encounters with C7 increased. These results indicate that hybridization between C7 and C9 is not possible and cannot be driving the decline of C9 in North America and C9 females suffer a significant fitness cost from mating with C7. Due to the rarity of heterospecific mating in these

species under extreme laboratory conditions, it is unknown how common the behavior is in the wild. However, the finding that C9 individuals are less likely to mate conspecifically after repeated non-sexual encounters with C7 is a very interesting outcome and has been identified by others as a potential cost to the evolution of satyrization-resistance (i.e., avoidance of mating with heterospecific). This has important implications for declining native species. C9 encounters with C7 are expected to increase as C7 proliferates and C9 declines. If C9 becomes less likely to mate as a consequence of increased interactions with C7, even without attempted copulation, already diminished populations may have little chance of recovery.

KEY WORDS: *Coccinella novemnotata*, native decline, satyrization, reproductive interference, introduced species

INTRODUCTION

Interspecific mating attempts in coccinellids in the field and laboratory have been occasionally observed in the genera *Adalia*, *Chilocorus*, *Coccinella, Exochomus*, *Harmonia*, *Propylea*, and *Synharmonia*, most often between congeners. The results of such copulations have been variable with females producing no eggs that show development, some eggs that develop but do not hatch, or very rarely, viable larvae (Ireland et al. 1986; Majerus 1997; Nedved & Honek 2012; Smith 1966; Webberley et al. 2004). In the extremely rare event that a true hybrid is produced, it will most likely be sterile, as was the case with a cross between a male *Adalia decempunctata* and female *Adalia bipunctata* (Majerus 1997). Snyder and Evans (2006) noted that *C. septempunctata* will mate with *C. transversoguttata,* but females of neither species produced fertile eggs. This behavior has been confirmed elsewhere, but with one notable difference – out of several copulating pairs a single *C. transversoguttata* female produced a few eggs that showed development, but did not hatch (Chapter 4, this volume). Interspecific mating attempts between *C. septempunctata* and *C. novemnotata* have never been reported in the literature. It is important to note that hybridization can lead to extinction of a rare species even in the absence of introgression (i.e., gene flow through repeated backcrossing of hybrids; Rhymer & Simberloff 1996). In fact, the production of viable hybrids is not necessary to significantly reduce the fitness of females that mate with a heterospecific male. In the coccinellid species *Henosepilachna vigintioctomaculata* and *H. pustulosa*, females experience a significant reduction in hatching ratios after only a single copulation with a heterospecific male precedes copulation with a conspecific (Katakura 1986).

The purpose of the present study was to document interspecific mating attempts and their outcomes between *C. septempunctata* and *C. novemnotata* to determine if hybridization is

possible and quantify the behavior by measuring the frequency of heterospecific mating attempts, latency to copulation (i.e., time lapsed to copulation start), and copulation duration. I assessed the fitness cost of heterospecific copulation on *C. novemnotata* females by subsequently mating them with a fertile conspecific male and comparing egg parameters before and after. I also determined if lack of access to opposite-sexed conspecifics influences propensity to mate with an opposite-sexed heterospecific. The 'mate deprivation hypothesis' predicts that males will be increasingly likely to engage in sexual coercion when access to females is limited (Thornhill & Thornhill 1983). In fact, a reduction in choosiness toward conspecifics after restricted mate access has been observed in a variety of organisms such as spiders (e.g., Wilder & Rypstra 2008), insects (e.g., Ortigoso & Rowe 2003) including coccinellids (e.g., Harmon et al. 2008), and humans (McKibben et al. 2008). In closely related species that occupy the same niche, if conspecific females are rare, is it possible that males will be more likely to mate with and/or sexually coerce heterospecific females? This may be the case in the forced copulation of the king penguin, *Aptenodytes patagonicus*, by its opportunistic predator, the Antarctic fur seal, *Arctocephalus gazella* (Charbonnier et al. 2010), an interaction that can severely injure the penguin (De Bruyn et al. 2008) or even result in its death (Haddad et al. 2014). Although the sex of the penguins were never determined, it is not relevant if the misdirected copulation was a function of mate-deprivation and not an error in species recognition. This is the most probable explanation given the high rates of fur seal population increase at Marion Island where this behavior has been observed, a phenomenon that may leave many males without access to suitable mates (Hofmeyr et al. 2006). To test the effect of mate-deprivation on the frequency of interspecific mating attempts between native and adventive coccinellids, I introduced pairs of *C. novemnotata* and *C. septempunctata* repeatedly over the course of several days to determine if their propensity to copulate increases in the prolonged absence of conspecifics and access to only

heterospecifics.

Finally, I wanted to know if non-sexual interactions with only heterospecifics imposes a fitness cost to individuals that avoid interspecific copulation (i.e., satyrization resistant) through reduced propensity to mate with a conspecific. In assays using *Aedes aegypti*, females that were resistant to satyrization by males of the congener *A. albopictus* were less likely to mate with conspecific males after non-sexual interactions with only heterospecific males (Bargielowski & Lounibos 2014). A "satyr" has been previously defined as a male that successfully mates with and consequently reduces the reproductive success of a heterospecific female (Ribeiro & Spielman 1986); the act is known as satyrization and the female is said to have been satyrized (Ribeiro 1988). The male is traditionally labeled the satyr because the act is expected to have a greater negative impact on females due to differential investment in gametes and thus reproductive potential. It is therefore usually assumed that an "error" in mate choice burdens males less than females (Parker 1982). However, this may not always be the case and same-sex sexual encounters between males can constitute satyrization if at least one male suffers a fitness cost (see review by Bailey & Zuk 2009).

Satyrization has been observed in a wide variety of species, including plants (Burdfield-Steel & Shuker 2011). Although most costs associated with satyrization do not usually put the satirized individual's life in danger, they can be quite substantial for the population. In fact, a model developed by Ribeiro and Spielman (1986) predicts the extinction of the satyrized species when a geographic barrier that once separated allopatric species is removed. This prediction holds even if interspecific mating attempts are rare as long migration constants are high (stable parapatry is predicted if migration is low). Due to this potential, Ribeiro (1988) proposed the use of satyrs in the biological control of insect pests and vectors. If there is a fitness cost, we would predict selection to favor satyrization-resistance in the satyrized species. Indeed, satyrization-

resistance has been documented for *Aedes aegypti* against *Aedes albopictus*, but only when individuals come from sympatric populations. *A. aegypti* individuals that are allopatric to *A. albopictus* are satyrization-susceptible, however, resistance can evolve rapidly, in as little as 1-3 generations after exposure to and satyrization by *A. albopictus* (Bargielowski et al. 2013; Bargielowski & Lounibos 2014). I therefore postulated that *C. novemnotata* or *C. septempunctata* individuals who refuse to mate with a heterospecific might already be resistant to satyrization. If resistance comes with a cost of reduced propensity to mate with a conspecific, we should see a decrease in conspecific mating attempts by *C. novemnotata* and *C. septempunctata* individuals who interacted, but did not mate, with a heterospecific. I tested this for both species, measuring the propensity to mate with a conspecific after zero to six interactions with a heterospecific and evaluated the number of copulating pairs.

MATERIAL AND METHODS

Specimens. All lady beetles originated from laboratory-reared colonies that were started with field-collected individuals from Colorado, Oregon, and South Dakota in 2009 and Oregon in 2012. Adults were maintained in cages containing potted fava bean plants (*Vicia faba*) infested with pea aphids, *Acyrthosiphon pisum* and kept at 22-23ºC with a 16:8 L:D cycle (similar to Hesler et al. 2012). Egg clusters were collected daily and placed in 44 ml plastic cups. After hatching, first instar larvae were given an *ad libitum* supply of pea aphids until first molt. Second-instar larvae were separated into 44 ml cups, reared individually, and fed pea aphids daily until pupation. Adult beetles were maintained individually in individual cups and fed pea aphids daily. Because male *C. septempunctata* (C7) and *C. novemnotata* (C9) have a refractory period after eclosion that lasts up to 8 d (Ugine & Losey 2014), C7 females take 11.2 d to reach sexual maturity post-eclosion (Omkar & Srivastava 2002), and adults separated for at least 2 weeks are more likely to mate with a heterospecific (Majerus 1997), adults in this study were

used in trials when they were 14 d post-eclosion to maximize propensity to copulate.

Propensity to mate - conspecific. To determine how often copulation occurs between conspecifics, 29 pairs of C9's and 32 pairs of C7's were introduced to each other and allowed to interact for up to 2 h. Males and females were paired according to age post-eclosion with ages being as close as possible. Pairs were placed in individual 163 ml lidded plastic cups and monitored until beetles made contact by touching or running into each other; from that point their behavior was checked every 5 minutes. Individuals that attempted to copulate were examined closely for insertion or attempted insertion of the male sipho. Only confirmed attempts (insertion of sipho for at least one five-minute interval) were counted as "successes" because males mounting with no attempt to copulate and females mounting males have been frequently observed in the lab (L.S., personal observation). The time interval at which copulation was first observed was noted and used to calculate the maximum latency to copulation initiation. If copulation occurred within the first 5 minutes, the latency to copulation was designated as 5 minutes. Trials were terminated at the end of 2 h or when the male dismounted for pairs that copulated longer than 2 hours. If a pair copulated intermittently, the trial continued until the end of the 2 h period or until the male dismounted after the end of the 2 h period. At the end of 2 h, mated females were isolated into 44 ml cups under the same conditions as before (16L:8D at 22-23ºC) and their eggs collected for 5 days. The time for C7 and C9 egg development at 25ºC is 3.0 d (Ugine & Losey 2014). Because eggs were held at a cooler temperature they were given additional time to develop, eggs from conspecific copulations were observed daily for development and hatching for 6 days. Copulation duration, latency to copulation, proportion of pairs that copulated, and proportion of females for which successful larval development occurred (at least one larva hatched) were recorded. For all trials, beetles were used only once.

Propensity to mate - heterospecific. To determine how often copulation occurs between C9 and C7, 46 pairs (C9♂xC7♀, n = 24; C7♂xC9♀, n = 22) were introduced in the same way as conspecific pairs. Hybrid coccinellid embryos have been shown to develop abnormally and asynchronously even when some viable larvae are produced (e.g., Katakura & Sobu 1986). To allow for unpredictable developmental delays eggs resulting from heterospecific copulations were observed for 8 days. Copulation duration, latency to copulation, proportion of pairs that copulated, and proportion of females for which successful larval development occurred (at least one larva hatched) were recorded.

Statistical analyses of propensity to mate. The proportion of trials that resulted in successful copulation for each of the three combinations: 1. C7xC7 vs C9xC9; 2. C7xC7 vs C7xC9; and 3. C9xC9 vs C7xC9 (note that the two versions of heterospecific crosses were combined to yield one C7xC9 group) was analyzed with a nominal logistic model that calculates a chi-square statistic for each comparison. The proportion of trials that resulted in successful copulation for the two conspecific conditions (C9xC9 and C7xC7) were pooled and compared to the heterospecific condition (C7xC9) using a nominal logistic model. The effect of species pair on copulation duration and maximum latency to first successful copulation attempt was tested using an ANOVA. Individual means were separated using a Tukey's HSD test. The two conspecific conditions were pooled and compared to the heterospecific condition using t tests for both parameters. The number of copulations that resulted in the production of at least one hatched larva was analyzed using a nominal logistic model for all pairwise combinations in addition to a test of the combined conspecific versus the heterospecific condition. Because propensity to mate trials were conducted over the course of three years, "year" was included as an interaction term and was dropped from the model if not significant.

Effect of heterospecific copulation on future reproductive behavior. To determine the impact, if any, mating with a heterospecific male had on egg viability after later mating with a conspecific, eggs were collected from C9 females (n = 21) that copulated with a C7 male for 5 days starting the day after mating. Following egg collection on the fifth day, females were paired with a conspecific male whose fertility had been previously confirmed by having mated with a virgin conspecific who laid viable eggs after mating with him. C9 pairs were allowed to interact for 2 hours. If a male refused to copulate, he was replaced with another fertile male until the female successfully copulated. This process was repeated as needed until all females copulated with a conspecific male. Eggs were collected for five days thereafter. The number of eggs laid, cannibalized, hatched, and showed signs of development but did not hatch was determined and compared to the data for the same females from the five days following the heterospecific mating. A separate group of virgin C9 females (n = 20) mated with fertile C9 males to serve as a control. The same data were collected for this group and the number of eggs laid, cannibalized, hatched, and showed signs of development but did not hatch. To determine if mating with a heterospecific female influences future male reproductive behavior, C7 (n = 27) and C9 (n = 26) males from a separate experiment (next section) were given access to a virgin conspecific female 2 d after mating with a heterospecific female.

Statistical analyses for effect of heterospecific copulation on future reproductive behavior. For females that mated with both a heterospecific and conspecific the total number of eggs laid, total number of eggs cannibalized, and total eggs hatched from the 5 days following the heterospecific copulation but prior to the conspecific copulation ("pre") were compared using paired two-tailed t-tests to the eggs collected after the conspecific copulation ("post"). The same comparisons were made between the control females and egg data from heterospecific-mated females after they mated with a conspecific ("post") using two-tailed t-tests. The proportions of

eggs that hatched and were cannibalized for "pre" and "post" conditions in heterospecific-mated females were evaluated using a binomial logistic regression. The same analysis was done to compare proportions for the "post" condition to the control females. The proportion of males that copulated a conspecific female after mating with a heterospecific was compared to the proportion of males that copulated with a conspecific female in the control group from the experiment described in the section "Control group: effect of time on propensity to mate with a conspecific" using a binomial logistic regression. The same analysis was used to compare the proportion of conspecific copulations between C7 and C9 males.

Effect of non-sexual interactions with a heterospecific on propensity to subsequently mate with conspecific. To determine if non-sexual interaction with only heterospecific beetles influences the likelihood that an individual will mate with a conspecific in the future, C9 males and females were introduced to an adult C7 of the opposite sex once per day for 1 hr at two-day intervals over the course of 12 d. Beetles were allowed to interact freely and observed for copulation. Pairs that copulated were removed from the pool of beetles and only individuals that did not mate were retained. These individuals comprised the treatment beetles that had interacted, but did not copulate with, a heterospecific. This process continued until there were groups of beetles that had the requisite number of interactions. There were six treatments for both species, defined by the number of non-sexual interactions with a heterospecific (H1 – H6) and for each treatment beetles were introduced to a conspecific the day after their last heterospecific interaction. For example, a beetle in treatment H3 interacted, but did not copulate with a heterospecific on days 1, 3, and 5 and on day 6 was introduced to an opposite-sexed naïve conspecific (from a separate pool of beetles use only for this purpose). The experiment began on day 0 with 194 heterospecific introductions (C7♀/C9♂ = 119, C7♂/C9♀ = 75; 388 beetles). Pairs were placed in individual 163 ml lidded plastic cups and observed for 60 minutes at 5-

minute intervals. Copulation was considered successful if a male inserted his sipho into the female and maintained that position for at least one time interval. To minimize bias due to unknown differential behavior of males and females between species (i.e., males of one species could be more likely to initiate heterospecific copulation), an attempt was made to ensure that as close to half of all trials involved a female C9 and male C7 and the other half involved a male C9 and female C7, but this was dependent upon beetle availability.

To more closely approximate conditions in the wild where the probability of encountering the same individual multiple times is assumed to be low, we never introduced the same individuals to each other during consecutive interactions. An effort was made to prevent repeated introductions throughout all 6 interactions, but this was not always possible because the pool of beetles rapidly decreased in size due to exclusion of beetles that mated with a heterospecific and because beetles from earlier treatments were never reused after interactions with a conspecific. If a heterospecific pair did not copulate, the pair was separated and once again isolated in individual cups until the next introduction two days later. Pairs that copulated for at least one time interval were removed from the experiment. This process was repeated until the last group of beetles had been exposed to a heterospecific 6 times over 12 consecutive days. Females and males that mated with a heterospecific were introduced to a conspecific beetle of the opposite sex were used in another experiment (see previous section "Effect of heterospecific copulation on future reproductive behavior**"**).

Control group: effect of time on propensity to mate with a conspecific. To account for the effect of time on the propensity to mate with a conspecific without any kind of interaction with a heterospecific, conspecific pairs of each species were introduced over the course of 9 days (C7) and 15 days (C9) with a two-day interval between each group. The first group of control beetles (day 1) also served as the first time interval of the experimental series (H0 = 0 interactions with a

heterospecific). Naïve beetles were used each time; beetles were not used again even if they did not copulate. There were 8 groups of C9 pairs (n = 20 pairs each day) and 5 groups of C7 pairs (n = 10 for groups 1-4, n = 14 for group 5).

Statistical analyses for non-sexual interactions with a heterospecifics. The proportion of heterospecific pairs that copulated as the number of interactions with a heterospecific increased (H0-H6) was evaluated using a nominal logistic model. The following tests were conducted for C7 and C9 separately in addition to comparisons between species using nominal logistic models: the proportion of conspecific pairs that copulated over time in each control group; and the proportion of conspecific copulations that occurred after beetles were exposed to, but did not mate with, a heterospecific as associated with the number of interactions (H0-H6).

To determine if propensity to mate with a conspecific was influenced by non-sexual interactions with only heterospecifics without including the variable of the number of interactions, the total number of conspecific pairs that copulated after all treatments (H1-H6, H0 was excluded because it did not involve a heterospecific) was pooled and compared to the combined number of conspecific pairs that copulated in all groups of the control for each species separately using a binomial logistic regression. Control males were pooled by adding all pairings over the course of the first 5 control introductions that spanned 10 days (C7, n = 69; C9, n = 115). This is appropriate because heterospecific-mated males were offered a conspecific female over the course of 11 days as determined by the outcome of their trial between Nov. 25 and Dec. 6, 2013.

Additionally, nominal logistic models were used to compare the total number of C7 and C9 males for all 6 treatments combined that copulated with a conspecific after first mating with a heterospecific female (C7 vs. C9) and against the number of males that mated with control females (each species vs. its control).

To test for consistency of results between experiments with respect to the frequency of heterospecific copulations, a nominal logistic model was used to compare the number of heterospecific copulations resulting from the first round of heterospecific introductions (H1) to the number of heterospecific copulations from the propensity to mate experiment (see "Propensity to mate – heterospecific"). Beetles from treatment H1 were used because they were the only naïve group used in the experiment and only naïve beetles were used in the propensity to mate experiment.

Data from all experiments were analyzed using JMP Pro (version 11.0 SAS Institute Inc.). A script for Grubb's Outlier Test (available from https://community.jmp.com/docs/DOC-6265) was used to evaluate the presence of outliers.

RESULTS

Conspecific and heterospecific mating behavior

Copulation incidence. A greater proportion of C9 pairs copulated (24/29) than both C7 (16/32; $p = 0.0061$) and C7xC9 pairs (9/46; $p < 0.0001$), and C7 pairs were more likely to copulate than C7xC9 pairs ($p = 0.0009$). Males of both species initiated heterospecific copulations equivalently (C7♂/C9♀ = 5, C9♂/C7♀ = 4). Conspecific pairs combined were more likely to copulate (65.6% of all pairs) than heterospecific pairs ($p < 0.0001$). Figure 1 shows the proportion of copulations per condition and the combined proportion conspecifics. There was no effect of "year" on copulation incidence for any comparison.

Copulation duration. C9 pairs copulated longer than C7xC9 pairs (C9: $\bar{x} = 153.33 \pm 8.76$; C7xC9 $\bar{x} = 70.56 \pm 14.18$; $p < 0.0001$), but not longer than C7 pairs (C7: $\bar{x} = 118.44 \pm 13.06$; $p = 0.060$). C7 pairs also copulated longer than C7xC9 pairs ($p = 0.042$). Pairs from the pooled conspecific condition copulated significantly longer than heterospecific pairs (pooled

conspecific: $\bar{x}$ = 139.38 ± 7.80; p = 0.0002; Figure 2).

Latency to copulation. There was no difference in the average maximum time elapsed to copulation or first attempt for any comparison (p = 1.0 for all multiple comparisons; p = 0.37 for conspecific vs. heterospecific; average time in minutes followed by the standard error of the mean; C7: $\bar{x}$ = 7.50 ± 1.21; C9: $\bar{x}$ = 8.75 ± 2.22; C7xC9: $\bar{x}$ = 7.22 ± 2.22; pooled conspecific: $\bar{x}$ = 8.25 ± 1.30).

Egg viability. Of the females that copulated with a conspecific 13 C7's and 18 C9's were used to determine the proportion of mated females that produced viable eggs. Roughly half of all conspecifically-mated C7 and C9 females produced viable eggs (C7 = 7/13, C9 = 10/18, p = 0.92; Figure 3). Females that mated with a heterospecific male always laid eggs but none showed signs of development. Because these females never produced viable eggs, both comparisons to conspecifically-mated females were significant (C7 vs C7xC9: p = 0.003 and C9 vs C7/C9: p = 0.0016; C7xC9 vs pooled conspecific: p = 0.001).

Effect of heterospecific mating on C9 egg production and viability.

Within-female comparisons (Pre- and post-conspecific). C9 females laid significantly more eggs after mating with a conspecific male than they did prior having mated only with a C7 male ("pre" = prior to conspecific, "post" = after; $\bar{x}_{pre}$ = 104.76 ± 11.46; $\bar{x}_{post}$ = 240.95 ± 18.17; p < 0.0001; Figure 4). There was no difference in the total number of eggs cannibalized before and after mating with a conspecific ($\bar{x}_{pre}$ = 11.38 ± 2.90; $\bar{x}_{post}$ = 13.71 ± 2.92; p = 0.78), however females cannibalized a significantly greater proportion of eggs prior to mating with a conspecific ($\bar{x}_{pre}$ = 0.11 ± 0.022; $\bar{x}_{post}$ = 0.060 ± 0.014; p < 0.0001). Only eggs laid after females mated with a conspecific hatched ($\bar{x}_{pre}$ = 0; $\bar{x}_{post}$ = 138.86 ± 21.28; p < 0.0001) and after mating with a conspecific, the average proportion of eggs hatching was 0.52 ± 0.067 (p < 0.0001).

Heterospecific-mated vs. Control Females. Control C9 females laid more eggs ($\bar{x}$ = 302.0 ± 15.92; p = 0.0016), cannibalized fewer eggs ($\bar{x}$ = 7.63 ± 2.60; p = 0.025), and had more eggs hatch ($\bar{x}$ = 217.94 ± 20.52; p = 0.0007) than C9 females that mated with a conspecific 5 d after mating with a C7 (Figure 5). Control females cannibalized a smaller proportion of eggs ($\bar{x}$ = 0.018 ± 0.004; $p < 0.0001$) and the proportion of control eggs that hatched was higher ($\bar{x}$ = 0.74 ± 0.032; $p < 0.0001$; Figure 5). One control female had an extremely low proportion of hatched eggs and was excluded from both hatching analyses and another control female cannibalized an unusually high proportion of eggs and was excluded from both cannibalized egg analyses.

Effects of repeated interactions with heterospecifics. There was no change in the proportion of heterospecific pairs that copulated as the number of introductions increased ($\bar{x}$ = 0.07 ± 0.0075; p = 0.22; Table 1). Over the course of 6 introductions a total of 52 heterospecific copulations occurred, 27 initiated by C7 males and 25 by C9 males, thus neither species was more likely to initiate a heterospecific mating.

There was no change in the proportion of conspecific pairs that copulated over time within each of the control groups (C7: $\bar{x}$ = 0.75 ± 0.022, p = 0.43; C9: $\bar{x}$ = 0.88 ± 0.024, p = 0.43; Table 2), but the difference between C7 and C9 was significant (p = 0.018).

There was no change in the propensity of C7's to mate with a conspecific after repeated non-sexual interactions with C9's (Table 3; Figure 6A; p = 0.24). However, C9's were significantly less likely to mate with a conspecific as the number of non-sexual interactions with C7's increased (Table 3; Figure 6B; p = 0.0022).

The proportion of C7's that mated with a conspecific after having been exposed to, but not mating with, a heterospecific at least one time was not different from the pooled control copulations (proportions: treatment = 0.86; control = 0.75; p = 0.13). In contrast, the proportion of C9's that mated with a conspecific under the same conditions was 31% significantly lower

than beetles in the control (proportions: treatment = 0.61, control = 0.88; $p < 0.0001$).

There was no difference between C9 and C7 males that had previously mated with a heterospecific female in their propensity to mate with a conspecific (proportions: C7 = 0.89; C9 = 0.96; $p = 0.31$). C7 and C9 males that mated first with a heterospecific female were just as likely to mate with a conspecific than control males (C7: $p = 0.1238$; C9: $p = 0.3041$).

The proportion of naïve heterospecific pairs that copulated in the first round of introductions (H1) was lower than in the propensity to mate experiment, but this difference was not significant (0.10 vs. 0.20).

DISCUSSION

Is hybridization possible between *C. novemnotata* and *C. septempunctata*? This study is the first to report that hybridization between *C. novemnotata* and *C. septempunctata* does not occur and, therefore, cannot be contributing to C9 decline in North America. There are three potential reasons why hybrids were not produced by C7 and C9 pairs that copulated, 1) differences in genital morphology (Hodek & Hoňek 1996) prevented transfer of sperm and/or spermatophore, 2) incapacitation of alien sperm in the female reproductive tract that either resulted in fertilization failure or the fertilization of eggs with weakened sperm (Katakura & Sobu 1986), and 3) death of hybrid embryos (Majerus 1997). Given the rarity of interspecific mating attempts, investigating the isolating mechanisms would not be relevant for conservation research, instead it would be interesting to determine what effect density has on the propensity to mate with a heterospecific. If a dramatic reduction in density leads to a greater probability of mating indiscriminately with heterospecifics and the individual incurs a fitness cost, interspecific mating attempts even without hybridization could pose significant consequences for a rapidly declining species.

Cost of mating with a heterospecific. Interspecific mating attempts were rare in this study and copulations lasting longer than the minimum time required for spermatophore transfer was even rarer. After copulating for 50 minutes 33% of C9 males are able to transfer a spermatophore, 71% after 60 minutes, and 100% after 70 minutes (Stellwag, unpublished data). Three out of the nine C9/C7 pairs that mated copulated for less than 30 minutes, therefore it is extremely unlikely that sperm was transferred by those males, if transfer is even possible between these two species. I could not dissect heterospecifically-mated females after copulation to check for the presence of a spermatophore because the females were needed for egg collection and/or additional assays, so I cannot confirm that the absence of visible embryonic development was due to lack of sperm or another reproductive barrier. Given that the average proportion of eggs that hatched from C9 females that mated with a C7 before mating with a conspecific was 30% lower than females that mated only with a conspecific suggests that heterospecific sperm was transferred and mixed with conspecific sperm. However, I cannot exclude the possibility that the reduction in egg viability was due to heterospecifically-mate females simply withholding sperm from a greater proportion of eggs. Inseminated females usually lay some proportion of unfertilized eggs in each clutch (Perry & Roitberg 2005) and there is strong evidence that this is a response to insufficient prey availability. The invasive lady beetle *Harmonia axyridis,* for example, increases unfertilized egg production by 56% when reared in a food-stressed environment (Perry & Roitberg 2005). Trophic egg production, or the intentional laying of unfertilized eggs to serve as food for larvae (Crespi 1992), has been documented in several coccinellids (e.g., Perry & Roitberg 2006; Osawa & Ohashi 2008). However, all beetles in the present study were reared with an *ad libitum* supply of aphids throughout the entire experiment so the significantly lower production of trophic eggs in the control females does not seem a reasonable explanation. The most probable explanation is that heterospecific sperm mixed with conspecific sperm and was used for egg fertilization by C9

females causing a significant fitness cost to C9 females that mate with a C7 male. Additional research involving the dissection of heterospecifically-mated females, however, is required to confirm this conclusion. It should be noted that all males engaged in the stereotypical side-to-side "wiggling" during copulation with a heterospecific female. This behavior is generally believed to be required for successful sperm transfer in some (Hodek & Hoňek 1996).

That heterospecifically-mated C9 females increased total egg production by 230% after mating with a conspecific implies that a critical behavioral or physiological stimulus was lacking during copulation with C7. Mating stimulates oocyte maturation and oviposition behavior in many insects (Chapman 2013; Gillott 2003), however virgin C7 females will start laying eggs as early as 8 days (T. A. Ugine, pers. comm.) and C9 as early as 6 days post-eclosion (Stellwag, unpublished data.). Due to the reliable production of eggs by virgins, oviposition by C9 females after mating with a C7 must not be interpreted as exclusively a reaction to copulation itself, they may have laid eggs at that time anyway, but the marked increase in egg production post-conspecific mating indicates that females adjusted their strategy either through withholding eggs or oosorption prior to mating with a conspecific. It is unknown what role oosorption (resorption of oocytes) plays in the reproductive strategies of most predatory lady beetles. The adaptive significance of oosorption, a highly plastic response to unfavorable conditions (i.e., lack of males) that occurs throughout the Insecta (Bell & Bohm 1975), has been studied in *Harmonia axyridis* (Osawa 2005) and the phytophagous lady beetle, *Epilachna niponica* (Ohgushi 1996), but the prevalence is unknown in other coccinellids. It is plausible that the greatly reduced fecundity of heterospecifically-mate C9 females as compared to their own egg production after mating with a conspecific and to that of the control females was due to increased oosorption. Why they laid fewer eggs after mating with a conspecific than control females cannot be ascertained. So, not only did C9 females incur a fitness cost in the form of reduced hatchability

of eggs after a single copulation with a C7, they also suffered a cost in the form of reduced fecundity. Either of these costs alone should be sufficient in selecting against C9 satyrization by C7 males.

C9 females that mated with a C7 cannibalized a greater proportion of eggs prior to mating with a conspecific. Filial cannibalism, or the deliberate consumption by parents of their young, is not predicted to be adaptive under most conditions. This probably explains why the behavior is extremely rare; yet, it is observed in some species, including insects (e.g., Bartlett 1987) although it does not always lead to larval death (e.g., Masuko 1986). Filial cannibalism of eggs by conspecifically-mated C7 and C9 females occurs frequently in captivity, even when food is available (Stellwag, unpublished data) and this may be an artifact of being confined to a small container. Even so, the significant reduction of egg cannibalism after mating with a conspecific suggests that the females possessed information about their fertility and adjusted their behavior accordingly. Additional research is required to understand why females that do not invest in offspring through post-zygotic parental care and have access to adequate food and sperm would ever engage in filial cannibalism.

Can costly interspecific mating attempts contribute to C9 decline? If the probability that a female C9 will mate with a C7 male increases as C9 density decreases due to mate-deprivation and C7 males are willing to initiate copulation with C9 females, interspecific mating attempts could potentially contribute to the decline of C9. The key here is that C7 males must be willing to initiate copulation. If C7 density increases, presumably so would the probability of finding a conspecific mate. If, as a result, these changes in abundance decrease the likelihood of C7 males initiating copulation with C9 females, then interspecific mating between C7 males and C9 females should be very rare. Additionally, if C9 males become more likely to mate with heterospecific females as a function of reduced density and increased encounter rate with C7

females, we still may not see a deleterious effect on a C9 population given that the propensity of heterospecifically-mated males to copulate with a conspecific female did not change in this study. Thus, the cost of interspecific mating between C7 and C9 appears to be asymmetric for the sexes, with females bearing most, if not all, of the cost. Sexual selection theory predicts that wasted reproductive efforts should be less costly for males than females (Parker 1982) and results from this study are consistent with that prediction, C9 females suffered a significant decrease in fitness through decreased fecundity and egg viability after mating with a C7 male, whereas C9 male behavior did not change, males of both species were just as willing to mate with a conspecific female after mating with a heterospecific. Unless some other fitness-reducing factor is present, C9 males are not likely to incur a substantial fitness cost from mating with C7 females.

The significant reduction in fitness for C9 females alone is sufficient to have a negative impact on C9 abundance even though heterospecific copulation was rare. The simulation model proposed by Ribeiro and Spielman (1986), dubbed "the satyr effect", demonstrates that reproductive interference via satyrization, a variable that is lacking from Lotka-Volterra models of competition, if present and operating simultaneously with resource competition, is likely to result in competitive displacement, possibly extinction, even at low frequencies of interspecific mating. The applicability of this model to C9 and C7, however, depends on migration between demes for each species, something that has not been studied because the satyr effect model predicts stable parapatry when migration between demes is low and extinction when high (unless equivalent for both species). The results from recent surveys on coccinellid communities combined with the thousands of submissions to the Lost Ladybug Project clearly indicate that C7 continues to increase its distribution across North America, while C9 appears to be confined to progressively smaller, remnant populations. This would suggest that migration could be high for

C7, but low for C9. If migration constants can be estimated for C7 and C9 in North America it could be interesting and potentially useful to predict the effect of C9 satyrization by C7 using the satyr effect model.

There are other potential costs associated with misdirected copulation besides wasted gametes, such as time lost and energy wasted that could be spent searching for appropriate mates or foraging, increased risk of predation (Gröning & Hochkirch 2008), and induction of refractory period (Nedved & Honek 2012). Conspecific C9 pairs copulated 30% longer than conspecific C7 pairs and in most cases, pairs were in copula for at least 2 hours, a time during which the female carries the male on her back. Females are mobile during this time, but they are slower and unable to fly. If disturbed, decoupling is not instantaneous, the male must retract his sipho, and thus, both individuals are handicapped during copulation. Another potential cost of unfruitful copulation attempts is the acquisition of sexually transmitted infections (Majerus 1997). There is limited evidence that transmission occurs interspecifically in coccinellids and no evidence that it could be driving native lady beetle decline in North America.

The cost of satyrization-resistance in *C. novemnotata.* A cost associated with satyrization-resistance in the form of reduced propensity to mate with conspecific has been confirmed in the yellow fever mosquito *Aedes aegypti* (Bargielowski & Lounibos 2014). Selection experiments that yielded individuals resistant to interspecific mating with the Asian tiger mosquito *Aedes albopictus*, showed those individuals to be less likely to mate with conspecific males (Bargielowski & Lounibos 2014). The authors found that exposing *A. aegypti* females to satyrization by *A. albopictus* males led to significantly reduced intraspecific mating rates after only a few generations, suggesting females with an evolved resistance to satyrization may be choosier than is adaptive. The gradual reduced propensity of C9 males and females to mate with a conspecific after a series of non-sexual interactions with a C7 suggests that C9 may experience

a similar cost. Interestingly, the same effect was not observed for C7.

Not all beetles were resistant to satyrization. The percentage of heterospecific pairs that copulated during each introduction was remarkably similar, between 5 and 10 percent, which indicates that a small set of beetles were satyrization-susceptible at all times. Among the 52 pairs that copulated 48% were initiated by C7 males indicating that males of neither species were more likely to initiate copulation. It is surprising, then, that only C9 individuals that resisted mating with heterospecifics were less likely to mate with a conspecific. The same general trend was present in C7, but it was not significant. This implies that C9 bears a cost to satyrization-resistance, but C7 does not. It is unclear why that would be the case. If this were due to reduced selective pressure against satyrization in C7 (i.e., lower cost for interspecific mating attempts by males), we would predict to see more C7 males than C9 initiating heterospecific copulations and this was not the outcome of the present study.

Although hybridization cannot be added to the list of negative interactions between C7 and C9 that may be driving C9 decline, reproductive interference through rare, but costly, interspecific copulation and a reduced propensity to mate with conspecifics due to evolved satyrization-resistance may be important. It is clear, however, that these behaviors alone cannot account for the ubiquitous dramatic decline in C9 populations in North America (Harmon et al. 2007), but could be acting synergistically with other documented antagonistic interactions such as exploitative competition (Hoki et al. 2014) and intraguild predation (Turnipseed et al. 2014; Tumminello et al. 2015). None of these interactions account for the fact that C7 females show higher reproductive potential compared to C9. In a comparative study on development times Ugine and Losey (2014) determined that the net reproductive rate for C9 was up to 50% lower than that of C7 due to the fact that C7 develops faster and lays significantly more eggs at a faster rate. When all of this information is considered together, it becomes more difficult to reject the

hypothesis that C7 has contributed to the decline of C9.

Conclusion and implications for conservation. The impact of hybridization is very well represented in the literature, but the production of hybrid progeny is not required to impose a significant asymmetrical fitness cost. This is particularly true in species where the female mates only once (e.g., Nasci et al. 1989), if a female mates with a heterospecific male she effectively becomes sterilized. In others species, attempting copulation is not even necessary to cause significant interference. For example, male seed-eating bugs, *Margus obscurator* (Heteroptera: Coreidae), indiscriminately sexually harass (through chasing, grappling, and mounting) other males, heterospecific females, and even beetles and bees (McLain & Shure 1987). The "victim" of this misdirected courtship typically flees in response, abandoning high-quality foraging and oviposition sites (McLain & Pratt 1999). Thus, a species prone to indiscriminate courtship can play a significant role in species exclusion even in the absence of exploitative resource competition. If this type of antagonistic interaction, as well as other forms of reproductive interference, is overlooked, we will form an incomplete picture of the forces driving not only reproductive character displacement, but also the interspecific interactions shaping ecological communities.

It is important to keep in mind that satyrization is not required for reproductive interference to have a negative impact and while clearly harmful to some native species in certain contexts, it may have the potential to be used as a weapon against economically important pests or even invasive species. Exploiting reproductive behavior to suppress pest populations is not a novel idea, the release of sterile males to interfere with female reproduction has been used in IPM programs for many years to combat agricultural pests (e.g., Krafsur 1998) and invasive species (e.g., Bergstedt & Twohey 2007). The introduction of heterospecific species to cause reproductive interference in a pest species may yield favorable results, especially if males prefer

heterospecific to conspecific females. Preference for heterospecific females has been observed in the invasive herbivorous mite, *Tetranychus evansi* (Sato et al. 2014) and squash bug, *Anasa andresii* (Hamel et al. 2015). Sadly, in both cases asymmetric mate choice appears to be harming or most likely to harm the native species with no apparent cost to the invasive males and this may partially explain their success as invasive species. Some non-hybridizing sympatric species also show a preference for heterospecific mates (e.g., Hochkirch et al. 2007) and it is hypothesized that these interactions may be very important in the evolution of habitat partitioning.

Finding compatible species to use in IPM or invasive species management for induced reproductive interference is at least possible theoretically, but may be very difficult because many factors beyond propensity to mate and lack of viable hybrid production must be considered. These include habitat use, density, migration, and timing of seasonal emergence (Ribeiro 1988; Singer 1990; Hettyey & Pearman 2003; Gröning et al. 2007) as well as abiotic influences (Miller & Svensson 2014). Could it be possible to use reproductive interference to lower the pressure on a native species by asymmetrically reducing the fitness of an invasive species or suppress agricultural pests? So far, no candidate species have been identified, but this may change as our understanding of the various forms and significance of reproductive interference continues to expand.

Figure 1. *Coccinella transversogutta* showing the complete (top right) and disintengrated (top left) transverse elytral band. *C. novemnotata* (bottom right) and *C. septempunctata* (bottom left) photos provided for comparison. Photos submitted by Gail Starr (top and bottom right) and Julie Craves (bottom left) to the Lost Ladybug Project. Top left photo by L. Stellwag.

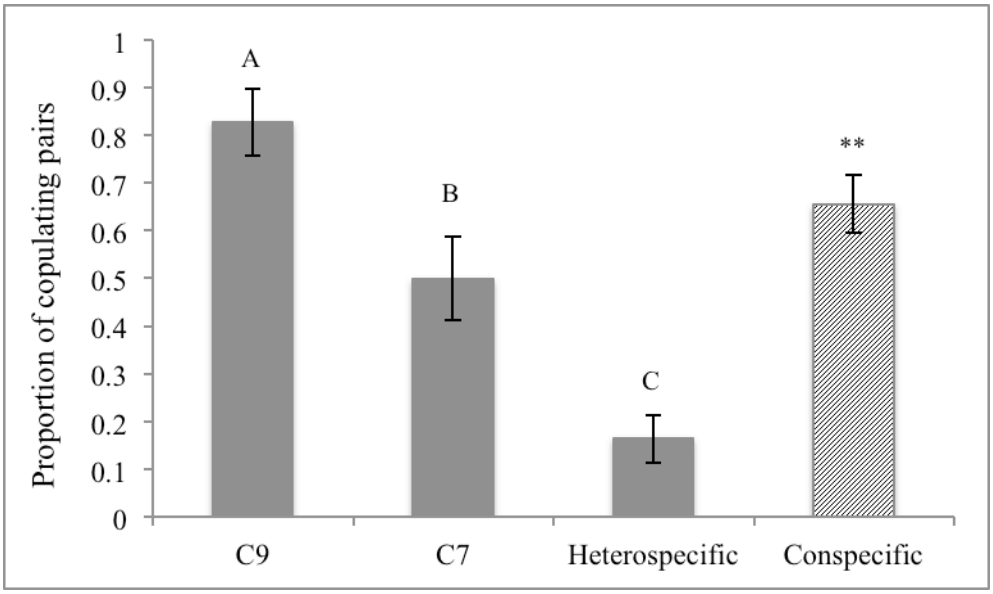

Figure 2. The proportion of trials that resulted in successful copulations for the two conspecific conditions separately and combined (Conspecific, pooled C7 and C9), and the heterospecific condition (C9xC7). The double asterisk above the conspecific bar indicates the significance of the test of the comparison to the heterospecific condition. Bars with the same letter were not different. Error bars represent the standard error or the mean.

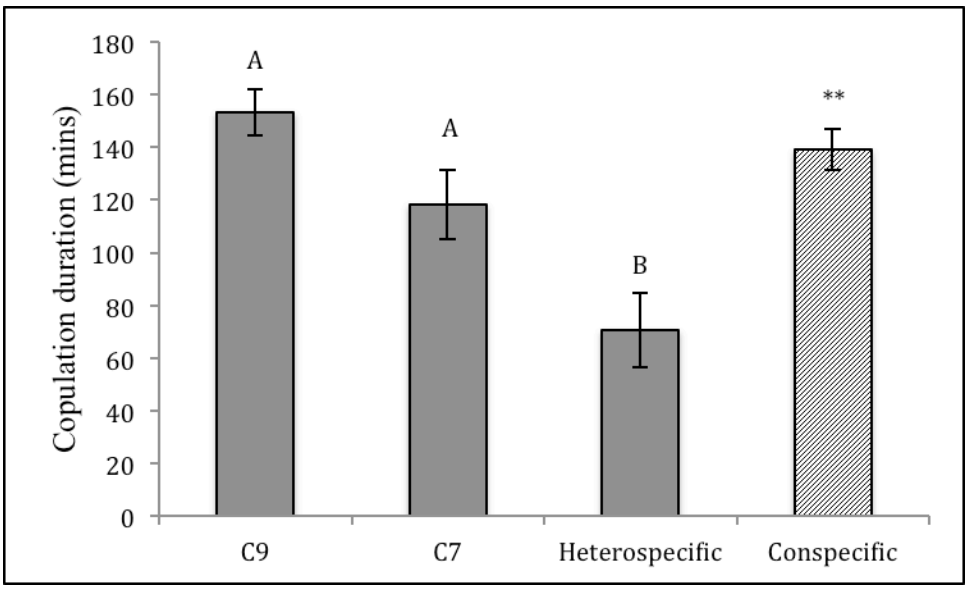

Figure 3. Average time, in minutes, lady beetle pairs spent copulating for the two conspecific conditions separately and combined (Conspecific, pooled C7 and C9), and the heterospecific condition. The double asterisk above the conspecific bar indicates the significance of the test of the comparison to the copulation duration of heterospecific pairs. Bars with the same letter were not different. Error bars represent the standard error of the mean.

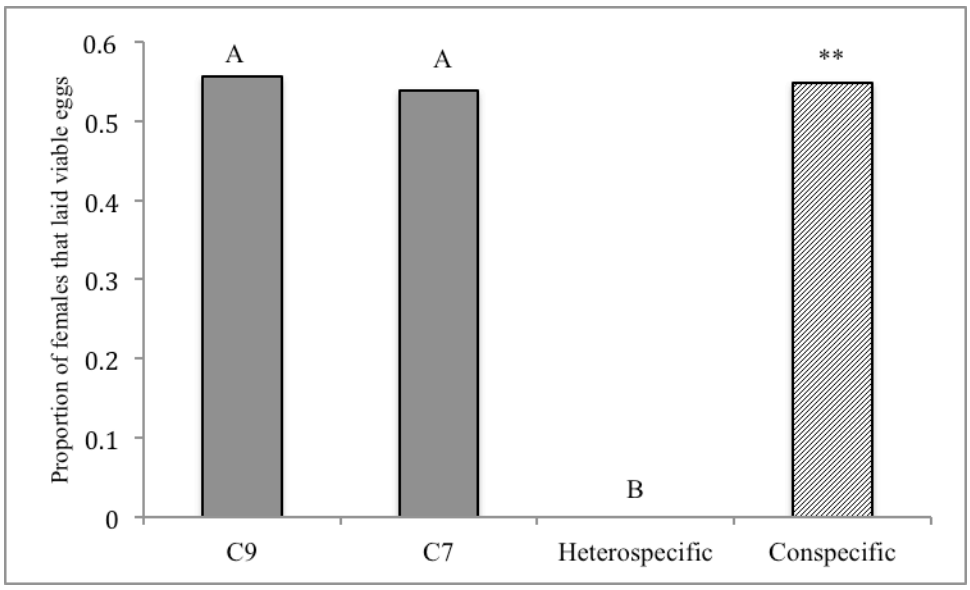

Figure 4. Proportion of mated females that produced viable eggs after copulation for the two conspecific conditions separately and combined (C7 and C9), and the heterospecific condition. Females that mated with a heterospecific male never produced viable eggs. The double asterisk above the conspecific bar indicates the significance of the test of the comparison to the proportion of heterospecific females. Bars with the same letter were not different.

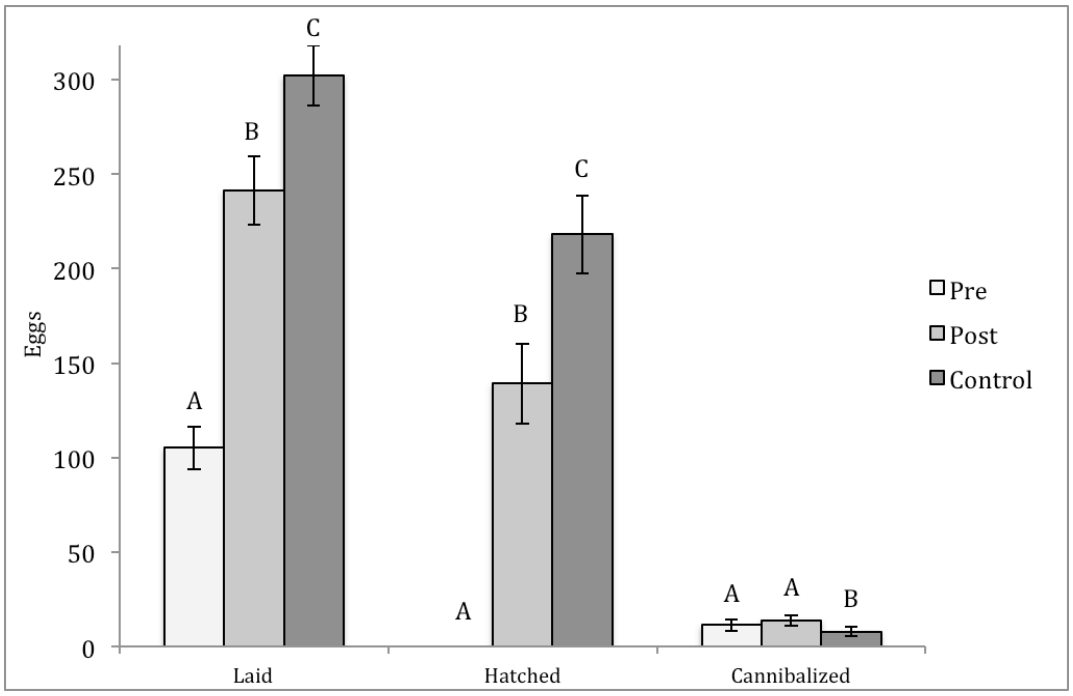

Figure 5. Total number of eggs laid, hatched, and cannibalized for C9 females that mated with a C7 before mating with a C9 ("Pre") and after ("Post") and C9 females that only mated with a conspecific ("Control"). Bars with different letters within each category were significantly different. Error bars represent the standard error of the mean.

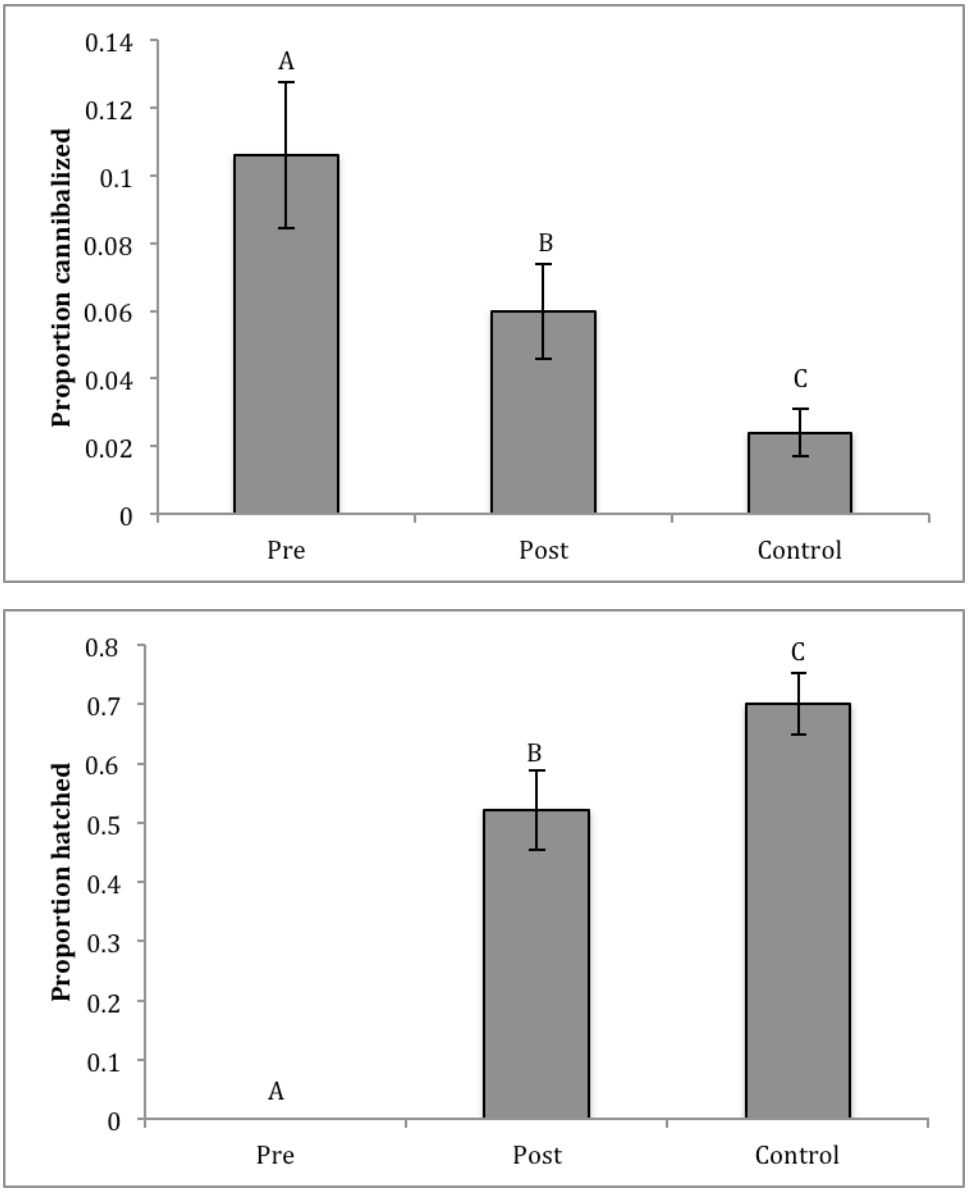

Figure 6: A (top): Proportion of cannibalized eggs. B (bottom): Proportion of hatched eggs for C9 females that mated with a C7 male (pre) and then mated with a conspecific (post) and C9 females that mated only with a conspecific (control).

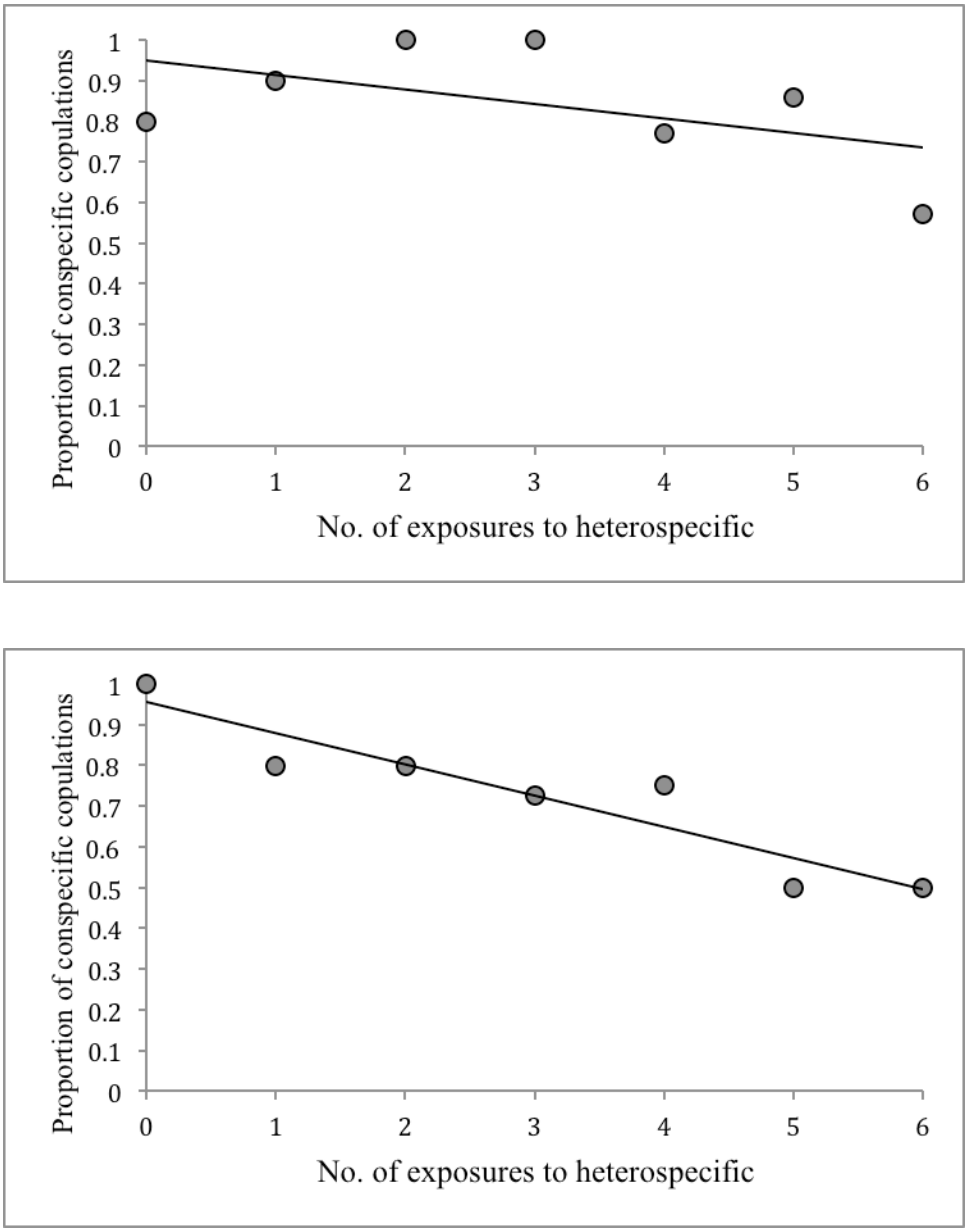

Figure 7: A (top). Proportion of C7 conspecific pairs that copulated after interacting, but not mating, with 0-6 C9's over the course of 12 days (2 days between introductions) with line of best fit. There was no effect of non-sexual interactions on proportion of conspecific copulations for C7. B (bottom). The same type of data for C9 conspecific pairs that copulated after being introduced to 0-6 C7's. There was a significant effect for C9.

Table 1. The number of heterospecific pairs for each round of interactions (intro #) in the heterospecific interaction experiment and the resulting number of copulations between *Coccinella novemnotata* and *Coccinella septempunctata*.

Intro #	n (pairs)	Copulating pairs	C7♂	C9♂	Prop cop
1	194	18	9	9	0.09
2	160	16	8	8	0.10
3	125	6	4	2	0.05
4	94	7	3	4	0.07
5	60	3	2	1	0.05
6	39	2	2	1	0.05
Totals	**672**	**52**	**27**	**25**	

Table 2. Controls for heterospecific interactions experiment. Conspecific pairs introduced over time and the resulting copulations.

Species	Time (d)	n	Copulating pairs	Prop cop
C7	0	15	12	0.8
	2	10	8	0.8
	4	10	8	0.8
	6	10	7	0.7
	8	10	7	0.7
	10	14	10	0.71
C9	0	15	15	1
	2	20	17	0.85
	4	20	17	0.85
	6	20	17	0.85
	8	20	19	0.95
	10	20	19	0.95
	12	20	16	0.8
	14	20	16	0.8
	16	20	18	0.9

Table 3. Conspecific introductions and the resulting copulations after 0-6 interactions with only a heterospecific over time.

Species	#Het interactions	n	Copulating pairs	Prop cop
C7	0	15	12	0.80
	1	10	9	0.90
	2	10	10	1.0
	3	10	10	1.0
	4	13	10	0.77
	5	7	6	0.86
	6	7	4	0.57
C9	0	15	15	1.0
	1	10	8	0.80
	2	10	8	0.80
	3	11	8	0.73
	4	12	9	0.75
	5	8	4	0.50
	6	6	3	0.50

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CHAPTER 4

ALLOPATRIC VERSUS SYMPATRIC INTERSPECIFIC MATING ATTEMPTS IN NON-HYBRIDIZING COCCINELLIDS: IMPLICATIONS FOR INTERACTIONS BETWEEN NATIVE AND INVASIVE SPECIES

Abstract

Three congeneric coccinellid species were used to test the hypothesis that heterospecific mating is more likely to occur in closely related allopatric than sympatric species due to lack of recent interactions to reinforce isolating barriers. The seven-spotted lady beetle, *Coccinella septempunctata* (C7) is sympatric with *C. transversoguttata* (CT) in Eurasia, but allopatric with *C. novemnotata* (C9) in North America. CT is native in both Eurasia and North America and is thus, also sympatric with C9. This design generated two sympatric pairings, C9/CT and C7/CT, and one allopatric, C7/C9. Naïve virgin adults were paired with an opposite-sexed heterospecific and allowed to interact. The proportion of copulating pairs was combined for the sympatric conditions and compared to the allopatric condition. Eggs resulting from heterospecific copulations were kept and observed for development. No eggs hatched, but one CT female that mated with a C7 male produced several eggs that showed incomplete larval development. The number of heterospecific pairs that copulated was low (6.1%), but more than half were between the allopatric C7/C9 pairs. Additionally, C7 males initiated a significant proportion of all heterospecific copulations (68%). Thus, C7 may have a greater negative impact through reproductive interference on allopatric native species. These results suggest that recent evolutionary relationships may be helpful in predicting and explaining the impact of introduced species on natives and should be considered in biological control programs prior to introductions.

KEY WORDS: allopatry, reproductive interference, *Coccinella*, satyrization

INTRODUCTION

Reproductive barriers are predicted to evolve rapidly among sympatric species when the cost of intermating is high (see reviews by Noor 1999; Marshal et al. 2002). When the fitness cost of mating with a heterospecific is substantial (e.g., sterilization) reproductive interference, defined as any kind of interspecific interaction occurring during the process of mate-acquisition that results in a reduction of fitness for at least one of the species involved (reviewed in Gröning & Hochkirch 2008), can strengthen the mechanisms for prezygotic isolation through greater mating discrimination in only a few generations (e.g., Bargielowski & Lounibos 2014). Because of ample opportunities for such costly interactions, mate-recognition signals should be more divergent for sympatric species than closely related allopatric species (Dobzhansky 1940), resulting in fewer "mistakes". Thus, reproductive interference through intermating by allopatric species is predicted to be more common. Indeed, it has long been observed that allopatric species more readily intermate than closely related sympatric species (Ehrman 1965; Dobzhansky et al. 1968; McLain et al. 1986; Majerus 1997; Bargielowski et al. 2013) and this frequently imposes asymmetrical fitness costs (Dobzhansky et al. 1968; Lachaise et al. 1986; McLain et al. 1986; Bargielowski & Lounibos 2014). Consequently, when two congeneric species suddenly encounter each other in secondary contact zones after a long period of allopatry, as is the case in many intentional or accidental introductions, they may be more likely to intermate with each other than with their sympatric congeners.

The evidence for reproductive interference between native species and their adventive congeners is mounting from studies on diverse animal and plant taxa including wildflowers (Galen & Gregory 1989), spider mites (Takafuji et al. 1997; Sato et al. 2014), frogs (Pearl et al. 2005; D'Amore et al. 2009), geckos (Dame & Petren 2006), grasshoppers (Hochkirch et al. 2007), dandelions (Takakura et al. 2009; Matsumoto et al. 2010; Nishida et al. 2014), mosquitoes

(Tripet et al. 2011; Bargielowski & Lounibos 2014), and true bugs (Hamel et al. 2015). If interactions between two species involve an asymmetrical reduction in fitness or if one of the species involved is more abundant, they can impose significant costs to one species through reproductive interference and even lead to displacement of the more vulnerable species (Takafuji et al. 1997; Reitz & Trumble 2002; Westman et al. 2002; Dame & Petren 2006; Liu et al. 2007; Kishi et al. 2009; Crowder et al. 2010; Kishi & Nakazawa 2013; Sun et al. 2014). Such is the case with satyrization, a phenomenon that imposes a significantly higher fitness cost to one member, usually the female, through heterospecific mating (Ribeiro & Spielman 1986). Often overlooked as a potential factor in the decline of native species due to the perceived rarity of heterospecific copulations among non-hybridizing species, reproductive interference through satyrization, even if rare, can work synergistically with resource competition to influence the outcome of native-invasive species interactions (Crowder et al. 2011; Kishi & Nakazawa 2013). In fact, several models predict that under certain conditions even if heterospecific mating attempts are rare, satyrization is sufficient to cause extinction of one species if stable parapatry through habitat partitioning is not possible (Feng et al. 1997; Kuno 1992; Ribeiro 1988; Ribeiro & Spielman 1986; Yoshimura & Clark 1994). When two allopatric species share an ecological niche, partitioning may not be possible after establishment if the decline of the satyrized species is too rapid, a condition in which extirpation seems most likely.

A unique opportunity presented itself in North America when the exotic lady beetle *Coccinella septempunctata* (C7) became established in the 1970's after multiple intentional releases for biological control of agricultural pests (Angalet & Jacques 1975; Angalet et al. 1979). The establishment and subsequent widespread proliferation of C7 in the United States provides the opportunity to investigate the role of evolutionary relationships (allo- or sym-patric) in the occurrence of reproductive interference between native and introduced species. This is

possible because of the distribution C7 shares with the two congeneric species, *C. transversoguttata* (CT) and *C. novemnotata* (C9). C7 shares a wide and overlapping distribution with CT in its native range throughout Eurasia (Johnson 1910; Kovár 2005; Marin et al. 2010) but is allopatric to the North American endemic species C9 (Wheeler & Hoebeke 1995). CT on the other hand, is native in North America (Gordon 1985) as well as Eurasia (Kovár 2005), thus it is sympatric with both C7 and C9. That C7 shares a niche and frequently interacts with both an allopatric (C9) and sympatric (CT) congener in its North American range (Alyokhin & Sewell 2004; Kajita & Evans 2010) raises questions about the potential role of reproductive interference in the precipitous decline of both C9 and CT in North America over the past 30 years. Co-evolution of C7 with CT in Eurasia should mean they have strong species-recognition mechanisms (see review by Noor 1999). In contrast, C7 has only been in contact with C9 for up to approximately 30 years in some parts of North America, less in others (Gordon & Vandenberg 1991). Prolonged interactions between C7 and CT in Eurasia should mean that they are less likely to intermate provided North American populations of CT have not evolved to the point of no longer being synonymous with Eurasian populations. In this study, even though the CT specimens originated in North America they were considered sympatric with C7. It was assumed that North American and Eurasian CT's still constitute a single species, although preferably this would have been confirmed. CT was also considered sympatric with C9 due to co-evolution in North America. C7 and C9, on the other hand, were considered allopatric since their overlap in distributions is relatively recent. If 30 years is an insufficient amount of time for the evolution of satyrization-resistance (i.e., avoidance of heterospecific mating) between these species, then C7 and C9 are still functionally allopatric. It should be noted that if the cost of satyrization is high enough (e.g., sterilization), selection will act rapidly and can lead to resistance within only a few generations (e.g., Bargielowski & Lounibos 2014). The rate at

which resistance (or reinforcement) develops depends on many factors including mating system characteristics (Marshal et al. 2002) and patterns of gamete utilization (Howard 1999), both of which significantly factor into the fitness cost to the satyrized species. In particular, the number of times a female mates is critical for determining the rate at which resistance develops. If a female mates only once and that happens to be with a heterospecific male she is effectively sterilized by that single copulation (e.g., Bargielowski & Lounibos 2014). Male and female coccinellids are promiscuous (Nedved & Honek 2012) and female reproductive success increases with each subsequent mating (Omkar & Mishra 2005). Unless there are other fitness costs associated with the behavior, a single copulation with a heterospecific male by a polyandrous female could have minimal impact and thus, significantly prolong the time required for selection to favor satyrization-resistance. Comparisons with other studies must take this into consideration. Regardless of how quickly resistance evolves, if satyrization plays a significant role in the interactions between introduced and native coccinellids and contributes to the rapid decline in native populations, the impact will be greatest in the early stages after establishment (Tripet et al. 2011). Thus, timing is critical, at some point the effect of satyrization will be undetectable even though it may have significantly contributed to the exclusion of a native species.

In this study, the role of recent evolutionary relationships between congeneric species was evaluated as a predictor of reproductive interference through heterospecific mating attempts between allopatric and sympatric coccinellids. I predicted that sympatric pairs (C7/CT and C9/CT) should be less likely to intermate than allopatric pairs (C7/C9) due to greater reproductive barriers (e.g., pheromones, genital morphology, etc.) as a consequence of long-term interactions in the same ecological niche and conclude that reproductive interference should not be overlooked in future research on the decline of certain native species.

MATERIAL AND METHODS

Specimens. All lady beetles originated from laboratory-reared colonies that were started with field-collected individuals from Colorado, Oregon, and South Dakota in 2009 and Oregon in 2012. Adults were maintained in cages containing potted fava bean plants (*Vicia faba*) infested with pea aphids, *Acyrthosiphon pisum* and kept at 22-23ºC with a 16:8 L:D cycle. Egg clusters were collected daily and placed in 44 ml plastic cups. After hatching, first instar larvae were given an *ad libitum* supply of pea aphids until first molt. Second-instar larvae were separated into individual cups, reared individually, and fed pea aphids daily until pupation. Adult beetles were maintained individually in 44 ml cups and fed pea aphids daily. It is known for a few coccinellid species that if deprived of interactions between opposite sex conspecifics for a period of two or more weeks, individuals show less rejection behavior even to the point of mating with other species more readily than normal (Ireland et al. 1986; O'Donald & Majerus 1992; Majerus 1994, 1997). Therefore, to increase the probability of heterospecific copulation attempts naïve virgin adults were used in trials when they were 14 d old (post-eclosion) and trials were conducted in 2011, 2012, and 2013.

To determine if the introduced coccinellid *Coccinella septempunctata* is more likely to mate with its allopatric (*C. novemnotata*) or sympatric (*C. transversoguttata*) congener, virgin C7 adults were randomly assigned to receive a C9 or CT opposite-sexed partner. An attempt was made to balance the sex distribution for each combination such that males and females of all species interacted. This was critical for the detection of any differences in the behavior of the sexes within heterospecific pairs (e.g., C7 males may be more likely to initiate copulation with C9 females than C9 males are with C7 females). Heterospecific pairs were placed in individual 44 ml lidded plastic cups and monitored until beetles made physical contact, from that point their behavior was checked every 5 minutes. Individuals that attempted to copulate were examined

closely for insertion or attempted insertion of the male sipho. Only confirmed attempts (insertion of sipho) that lasted for at least 20 minutes were counted as "successes" because males mounting with no attempt to copulate or abandoning the female after a few minutes and females mounting males have frequently been observed in the lab (L.S., personal observation). If a pair did not make a successful copulation attempt after 1 h, the trial was terminated. Females that mated with a heterospecific male were isolated for egg collection and held for one week. Eggs were checked daily for development. A previous study determined that C7 and C9 never produce eggs that hatch or show development (Chapter 3, this volume), but the same result has not been reported for C7 with CT or C9 with CT. Nevertheless, eggs resulting from all heterospecific matings were inspected.

To test the hypothesis that sympatric species are less likely to mate than allopatric species, CT and C9 individuals were introduced to each other in the same manner as described above. This design yielded three groups of heterospecific pairs, one allopatric (C7/C9) and two sympatric (C7/CT and C9/CT) and a total of 918 beetles were used (459 pairs). The breakdown of trials per year is as follows: 2011 (n = 50), 2012 (n = 32), and 2013 (n = 377). Sample sizes for each species/sex combination are provided in Table 1.

Statistical analyses. Data were analyzed with JMP Pro (version 11.0 SAS Institute Inc.) (www.jmp.com). Nominal logistic models were used to determine 1) the overall effect of species combination (C7/C9, C7/CT, C9/CT) on the number of heterospecific copulations; 2) if pairs involving a C7 differed in outcome based on its relationship (allopatric: C9 or sympatric: CT) with the other individual; 3) the influence of population origin on the likelihood of interspecific mating by combining the number of copulating pairs from the two sympatric combinations (C7/CT and C9/CT) and comparing that to the number of copulating pairs from the allopatric combination (C7/C9); and 4) if males of each species behaves differently (i.e., males

of one species may be more likely to initiate copulation with a heterospecific female than the reverse configuration), and thus serve as a better predictor of trial outcome than species combination. This last analysis involved multiple comparisons therefore, to control experimentwise error from the chi-square comparisons, the results were interpreted using a Bonferroni correction (calculated *p* value x 3) and analyzed at $\alpha = 0.05$. Because trials were conducted over the span of 3 years, the interaction of main effect with "year" was evaluated for each model.

RESULTS

Out of 459 trials, 28 (6.1%) resulted in successful copulation attempts and at least one copulation resulted from each species/sex combination except CT♂/ C7♀ (Figure1; Table 2). The main effect of species combination on the likelihood of copulation was significant ($p = 0.037$). Approximately 57% of heterospecific copulations were between the allopatric pairs C7/C9. This species combination was significantly more likely to result in copulation than when C7 was paired with its allopatric congener CT (Figure 2; $p = 0.039$). The effect of origin also was significant with allopatric pairs (C7/C9) being more than twice as likely to copulate than pairs from the combined sympatric condition (C7/CT and C9/CT) (Figure 3; $p = 0.01$). C7 males initiated most copulations (roughly 68%; Figure 4; Table 2) and were significantly more likely to initiate copulation than males of both CT ($p = 0.001$) and C9 ($p < 0.001$). There was no difference in the behavior of C9 and CT males in the initiation of copulation ($p = 0.59$). There was no interaction between year and main effects in the analysis on species combination ($p = 0.37$) or origin ($p = 0.46$), but there was a significant interaction with year and species of male ($p = 0.027$). Eggs resulting from heterospecific matings never hatched, but one CT female that mated with a C7 male produced several clutches of eggs that showed clear signs of partial larval development (Figure 5).

DISCUSSION

That C7/C9 pairs (allopatric) were involved in significantly more mating attempts than the combined sympatric conditions (C7/CT, C9/CT) strongly suggests that there has been selective pressure on these sympatric congeners to avoid costly mate-choice errors. Only in sympatry can reproductive barriers evolve and in coccinellids this appears to be primarily chemical through species-specific cuticular hydrocarbons (e.g., Hemptinne et al. 1998). It appears that chemical signals alone, however, are insufficient for mate-recognition and that female behavior plays a critical role in the decision-making process of the male (Hemptinne et al. 1998). The experiments conducted thus far with C7, C9, and CT have not involved simultaneous choice of mates, but based on how readily conspecific pairs mate in the lab (Chapter 3, this volume) and how rare heterospecific copulations are, it seems unlikely that, if presented with a choice between a conspecific or heterospecific, there would be any hesitation in selecting the conspecific. It has consistently been my observation that if a male is going to attempt to copulate, that he will do so readily and typically upon first contact with the female. In fact, latency to copulation is no different between conspecific and heterospecific pairs (Chapter 3, this volume). This suggests that some males exposed to the artificial conditions of the lab (i.e., virgins deprived of females for 2 weeks) are eager to mate with whatever female is presented to them, something Majerus (1997) called "male randiness". Other researchers have reported a stereotyped series of behaviors by male *Harmonia axyridis* that seem to indicate assessment of females. The behaviors involve sequentially approaching, watching, and examining the female prior to mounting and making an insertion attempt (Obata 1987). In my experiments, all copulatory attempts by males involved mounting of the female immediately after contact, with no apparent hesitation, something Hemptinne et al. (1998) also observed in *Adalia bipunctata*. In fact, even among copulating heterospecific pairs the latency to copulation

as determined by male insertion was not different from conspecific males (Chapter 3, this volume). Based on these observations, it appears that if a deprived, virgin C7, C9, or CT male is going to mate, he will likely do so with any female and this may simply be an artifact. It is unknown how likely males are to mate with a heterospecific when given access to conspecific females and this should be taken into considering when evaluating the results of this study.

This study confirmed the inability of C7 and C9 to produce hybrids, is the first report of intermating between C9/CT and the second report for C7/CT (Evans et al. 2011). Additionally, this study provides the first evidence of a post-zygotic isolating barrier between C7 and CT due to the partial development of several eggs (Figure 5) indicating that sperm transfer and fertilization is possible in these two species. Sperm transfer between C7/C9 and C9/CT is still unknown.

The establishment of strong isolating barriers among heterospecific congeners of all species combinations was evidenced by the overall low proportion of heterospecific copulations (6.1%) and is consistent with my other research (Chapter 3, this volume). However, even when rare, heterospecific copulation can have significant deleterious consequences (Ribeiro 1988). The big question then is, just how costly is mating between congeners among *C. novemnotata*, *C. septempunctata*, and *C. transversoguttata*? In another experiment I demonstrated that C9 females incur a significant fitness cost in reduced fecundity and percent egg viability due to mating with a C7 male prior to mating with a conspecific (Chapter 3, this volume). The C7 males that mated with C9 females showed no decrease in propensity to mate with a conspecific after mating with C9. While the cost to males for mating mistakes is clearly not zero, generally the cost is substantially greater for females (Parker 1982). Male lady beetles that mate with inappropriate partners waste time that could be spent foraging or searching for conspecific females, risk exposure to sexually transmitted diseases and parasites (Hurst et al. 1995; Majerus

1997), and are at increased vulnerability to predation during copulation (Majerus 1997). The act of copulation itself, which lasts between 1-2 hours in C7 and C9 (Chapter 3, this volume), is energetically costly for many coccinellid males due to the vigorous bouts of side-to-side "shaking" that is thought to be necessary for sperm transfer in some species (e.g., Obata 1987), a behavior that is repeated about 200 times with 25 second intervals during a copulation (Hodek & Hoňek 1996). Additionally, males of many coccinellid species produce a proteinaceous spermatophore that females consume or absorb after sperm have migrated to the spermathecum (Obata & Hidaka 1987; Obata 1987; Obata & Johki 1991). The function of the spermatophore in lady beetles is not fully understood; experiments testing hypotheses about investment, sexual conflict, and signaling have yielded conflicting results (Omkar & Mishra 2005; Perry & Rowe 2008a, 2008b). Regardless of the function, the cost associated with spermatophore production and the effort required to transfer it clearly is not inconsequential and males who mate with a heterospecific waste valuable resources. Females incur many of the same costs (Daly 1978), but females of some coccinellid species also face reduced longevity with an increasing number of matings presumably as a tradeoff for increased fecundity and percent viability (Omkar & Mishra 2005; but see Omkar et al. 2010). Thus, mating with a heterospecific male could contribute to decreased longevity without the associated benefit of polyandry. Interestingly, Omkar and Mishra (2005) found no decrease in longevity for C7 and attributed it to spermatophore consumption, but this conclusion has been questioned (Perry & Rowe 2008b). Given the obvious costs to both sexes for mating with conspecifics alone, not accounting for any extra costs associated with misdirected attempts, individuals should be under strong selection to minimize mate-choice errors (Edward & Chapman 2011) and the only way selection can act is if the species are interacting regularly.

The two critical assumptions made in this study were supported by the result that C7/C9

pairs were more likely to mate than C7/CT (10% versus 4% of all introductions). First, this implies that the time spent by C7 interacting with C9 in North America since its establishment has not yet led to satyrization resistance, but this can only be stated for the areas in the western U. S. where the specimens for this study were collected (most of which were from Oregon). C7 has gradually expanded its distribution in the continental U. S. (Angalet et al. 1979) from east to west over the span of 22 years (Losey et al. *in preparation*) with the first indication of establishment in New Jersey in 1974 (Angalet & Jacques 1975). C7 was first detected in South Dakota in 1987 (Hesler & Kieckhefer 2008), Colorado in 1988 (Cornell University Insect Collection), and Oregon in 1996 (Losey et al. *in preparation*). Thus, western C9's have spent less time in shared habitats with C7 than eastern populations. This may account for the significant effect of year on the species of male initiating copulation. That Oregon was the last state in the continental U. S. to detect the presence of C7 may explain why 50% of all C7/C9 mating attempts happened in 2013. All beetles used in 2013 were from Oregon, whereas the previous years also included specimens collected from Colorado and South Dakota. This implies that C9 and C7 in Oregon are more susceptible to intermating, but that it should change over the next few years. The same assumption may not hold true for individuals from eastern parts of the U. S. where C9 was feared to be extirpated until recent rediscoveries (Stephens 2002; Losey et al. 2007, 2014). Therefore, we might see behavioral differences between C7 and C9 heterospecific pairs that originate from areas that vary in the length of time since C7 was first detected simply due to the availability of sufficient interactions. It would be interesting to use eastern C9's to see if they are more resistant to mating with C7. If that is the case, satyrization-resistance may help explain their persistence in areas where they were once abundant.

Second, the result that C7/CT copulations were rare, occurring between only 4% of C7/CT pairs, suggests that Eurasian and North American CT's have not significantly diverged

since they became isolated meaning C7 and North American CT's can be considered sympatric. Determining the outcome of mating between CT specimens from the two regions would be useful in confirming this. Even without this information, the present study provides strong evidence that allopatric species are more susceptible to reproductive interference than sympatric species and because of CT's greater resistance to satyrization, its decline in North America must be due to other factors such as resource competition. This is not the first observation of allopatric coccinellid species being more likely to intermate. In a paper on coccinellid hybridization, Majerus (1997) noted that among the 17 species studied there was a tendency for sympatric species to mate less readily than those without overlapping distributions and nearly all allopatric species that mated were congeneric. Although he did not use the same term, he attributed the phenomenon to satyrization resistance in species that share an ecological niche. The results of the present study corroborate findings from other taxa (e.g., Bargielowski & Lounibos 2014) and highlight the importance of including reproductive interference in the research about antagonistic interactions between invasive and native species. Biological control programs involving the release of non-native species should carefully evaluate evolutionary relationships with native congeners, especially if they share a niche and will, thus, interact on a regular basis. Adding reproductive interference to the list of concerns to be researched prior to new species introductions may help mitigate the impact felt by some indigenous species.

Conclusion. Research into the impact of invasive species is very important, not only for the obvious economic reasons (Iperti 1999; Snyder 2009), but also ecological consequences (Chapin et al. 2000). The potential role of introduced coccinellids, in particular *C. septempunctata*, in native lady beetle decline has received much attention over the last few decades (e.g., Harmon et al. 2007) and experiments by a variety of researchers have focused exclusively on resource

competition (e.g., exploitative competition, intraguild predation, habitat displacement). As the evidence mounts, confirming the presence of various antagonistic competitive interactions (e.g., Evans 2004; Losey et al. 2012; Hoki et al. 2014; Turnipseed et al. 2014; Tumminello et al. 2015), it is becoming more difficult to argue against the conclusion that C7 plays an important role in the decline of at least some lady beetles. However, it is important to consider all potential interactions that can differentially affect each species, not just resource competition. Reproductive interference alone, when rare, may not threaten a species, but when it occurs among species that also compete for resources and imposes asymmetrical fitness costs, it can work synergistically with competition and rapidly lead to the exclusion of a species (Ribeiro 1988; Crowder et al. 2011; Kishi & Nakazawa 2013). It is therefore critical that reproductive interference, especially through satyrization, be considered in situations involving allopatric congeneric species that share an ecological niche. Even if it is too late for the recovery of the native species, we may be able to get closer to understanding why the species was susceptible and use this knowledge to prevent the same fate for other species.

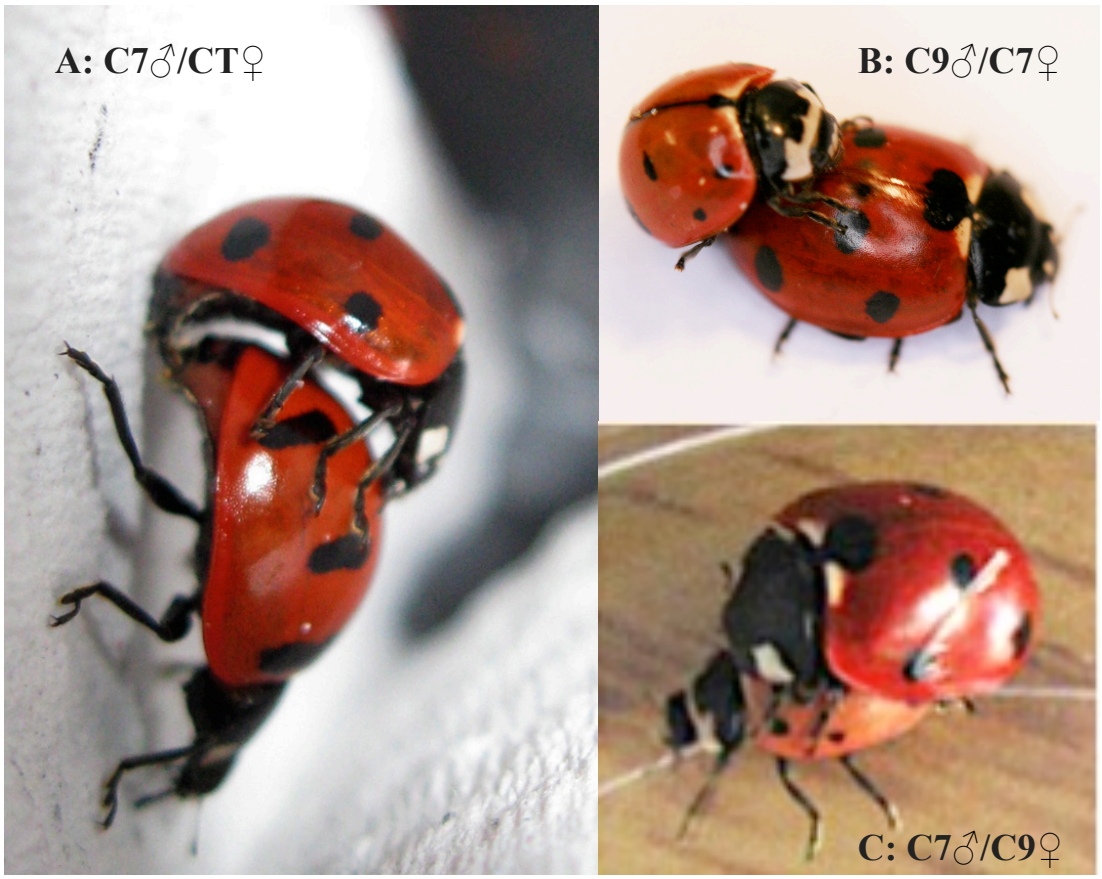

Figure 1: Interspecific mating between A) *C. septempunctata* ♂ and *C. transversoguttata* ♀, B) *C. novemnotata* ♂ and *C. septempunctata* ♀, and C) *C. septempunctata* ♂ and *C. novemnotata* ♀. Not pictured is *C. novemnotata* ♂ with *C. transversoguttata* ♀ or *C. transversoguttata* ♂ with *C. novemnotata* ♀. Photos by L. Stellwag.

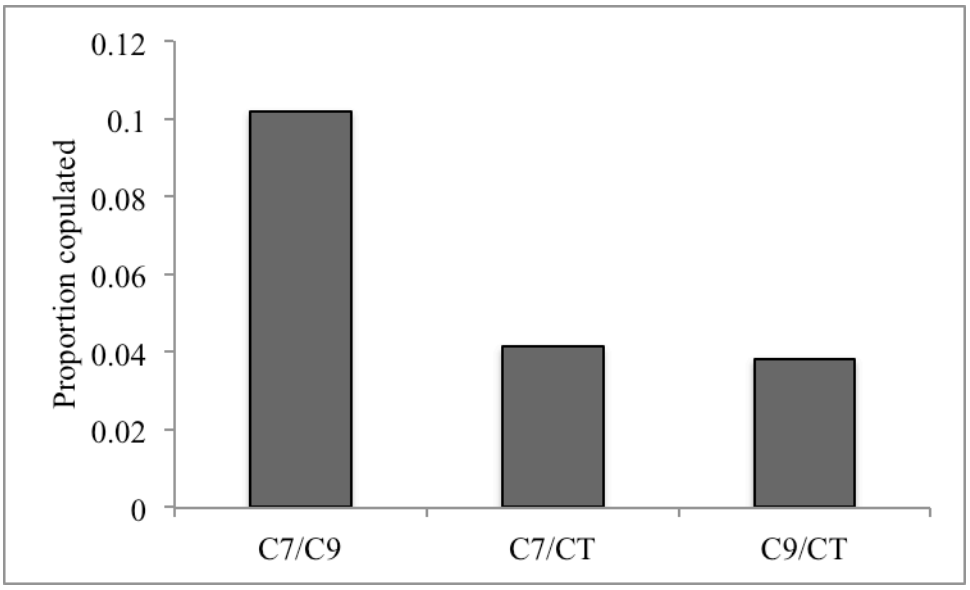

Figure 2. The proportion of heterospecific pairs that copulated for each species combination. There were a total of 28 copulating pairs out of 459 introductions. The adventive species, *Coccinella septempunctata* (C7) is allopatric with native *C. novemnotata* (C9) and sympatric with native *C. transversoguttata* (CT), while C9 and CT are sympatric. C7/C9 pairs accounted for 57% of all heterospecific copulations. See text for analyses.

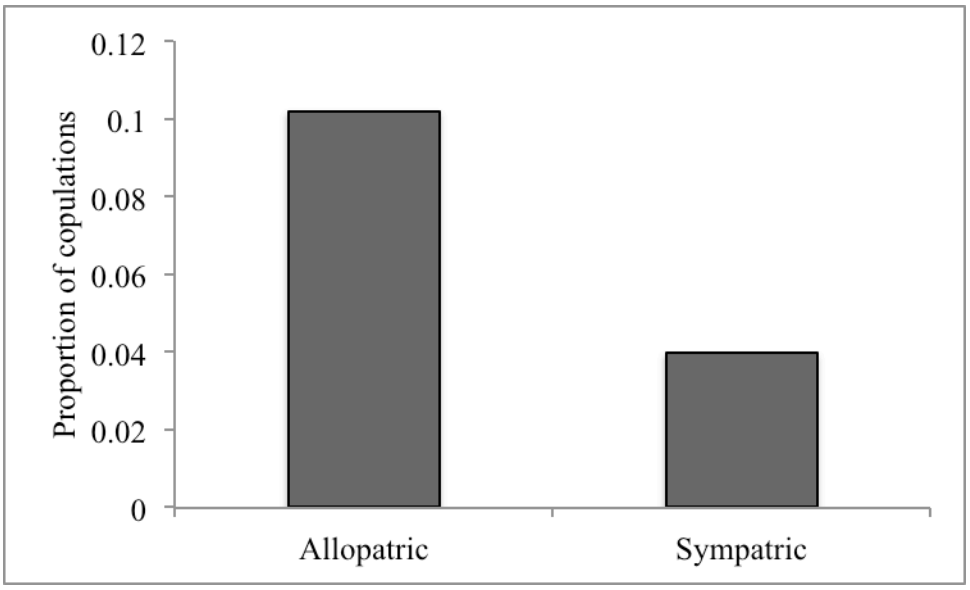

Figure 3. The proportion of trials resulting in heterospecific copulations between allopatric (C7/C9) and combined sympatric (C7/CT and C9/CT) pairs. Allopatric pairs were significantly more likely to mate ($p = 0.01$) than sympatric pairs.

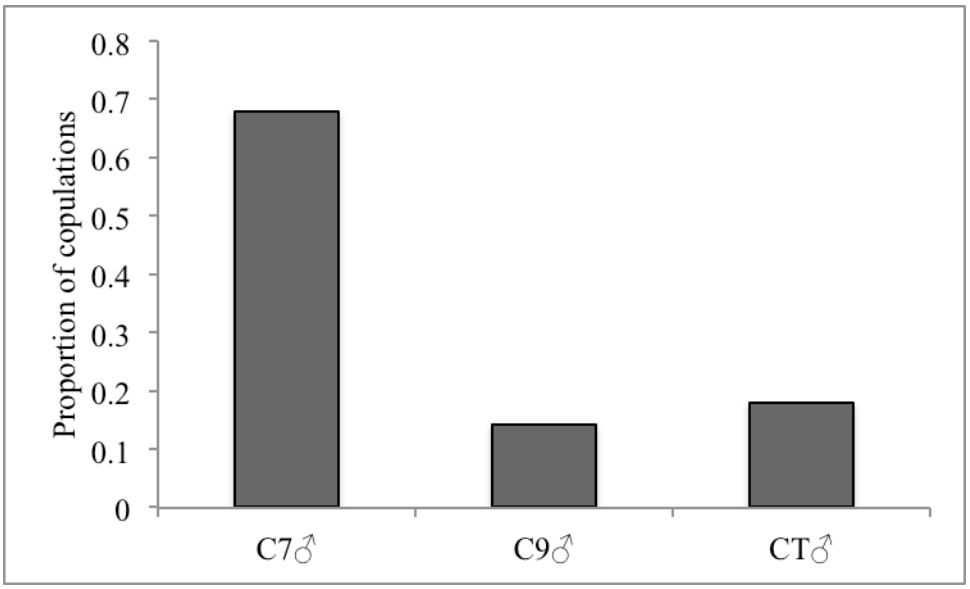

Figure 4. The proportion of heterospecific copulations initiated by males of each species, *C. septempunctata* (C7; introduced), *C. novemnotata* (C9; native), and *C. transversoguttata* (CT; native). C7 males initiated significantly more heterospecific copulations in general than C9 or CT ($p < 0.001$), more than half of which were initiated with C9 females.

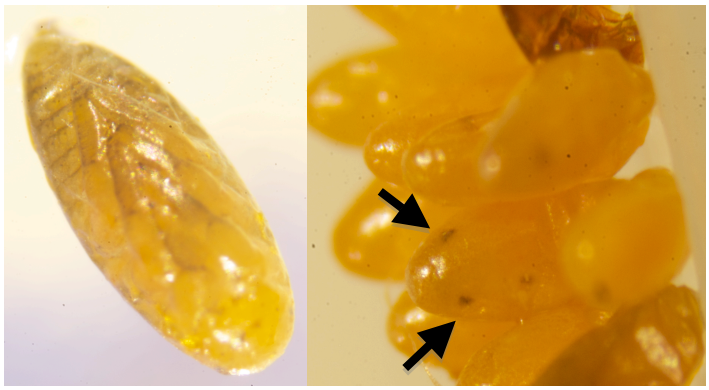

Figure 5: Eggs from a *Coccinella transversoguttata* female after mating with *C. septempunctata* showing larval development. Arrows point to eyes in the photo on the right. Several, but not all, eggs in each clutch showed signs of development. Development of sternites and legs are clearly discernable in the photo on the left. No eggs hatched. Photos by L. Stellwag.

Table 1: Sample sizes for each species combination for heterospecific pairs partitioned by sex. Virgin *C. septempunctata* (C7) males and females were paired with a virgin *C. novemnotata* (C9) or *C. transversoguttata* (CT) of the opposite sex. Total sample sizes were: C7/C9 = 157; C7/CT = 145; C9/CT = 157.

	C7♀	C9♀	CT♀
C7♂	------	67	70
C9♂	90	------	82
CT♂	75	75	------

Table 2: The number of copulations observed for each heterospecific pair by sex. There were a total of 28 successful copulations out of 459 introductions. See Table 1 for sample sizes for each pairing. The percentage of pairs that copulated out of all introductions for that combination is in parentheses.

	C7♀	C9♀	CT♀
C7♂	------	13 (19.4)	6 (8.6)
C9♂	3 (3.3)	------	1 (1.2)
CT♂	0 (0)	5 (6.7)	------

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